ZOOLOGY

(M. Sc. PROGRAMME)

SEMESTER-IV

ELECTIVE THEORY PAPER:

CELL AND DEVELOPMENTAL BIOLOGY

ZET-403

DIRECTORATE OF OPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

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Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

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Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

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Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE THEORY

CELL AND DEVELOPMENTAL BIOLOGY (ZET 403)

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 403 (Cell and Developmental Biology)	**I**	Nucleocytoplasmic interaction in early development; Importance and role of cytoplasm, biochemical evidence for functional state of genome, hybridization experiments, nature of changes in nuclei, cell hybridization and nuclear transplantation experiments	**2**	**1**	**1**	**5-22**
	II	Biological specificity : Transplantations and rejection		**1**	**1**	**23-35**
	III	Growth: Definition, Relative growth of parts, growth gradients		**1**	**1**	**36-49**
	IV	Regeneration: origin of regenerating cells and their potentialities, Field action in regeneration		**1**	**1**	**50-59**
	V	Proteins during development: Lens crystalline: Classification, ontogeny of crystalline in fish, chick and mammals		**1**	**1**	**60-63**
	VI	Hemoglobin: structure, heterogeny and ontogeny		**1**	**1**	**64-74**
	VII	LDH: structure, function, ontogeny, heterogeny, control of isozyme patterns		**1**	**1**	**75-82**
	VIII	Statistics in biology : Test of hypothesis: Chi-square test, Paired 't' – test Non-parametric tests: Spearman's Rank correlation, Wilcoxon Signed Rank test.		**1**	**1**	**83-95**

Unit I

Nucleocytoplasmic interaction in: In early development; Importance and role of cytoplasm, biochemical evidence for functional state of genome, hybridization experiments, nature of changes in nuclei, cell hybridization and nuclear transplantation experiments

Objective: In this unit you will know about Nucleocytoplasmic interaction in early development; Importance and role of cytoplasm, biochemical evidence for functional state of genome, hybridization experiments, and nature of changes in nuclei, cell hybridization and nuclear transplantation experiments

Nucleocytoplasmic Interaction

The cytoplasm is the centre of metabolic activities in the cell and the nucleus is the ultimate controlling centre. The cytoplasm provides the suitable environment in which the cytoplasmic as well as nuclear genes operate. Many enzymes operate through the cytoplasm and particularly through certain living cytoplasmic structures. Hence it can be expected that the cytoplasm exerts an influence on genie action.

In fact, the cytoplasm contains several hereditary units which are termed plasmogenes or cytogenes. These units which contain their own genetic material are believed to be self-duplicating and are capable of determining hereditary characters. The totality of hereditary factors transmitted through cytoplasm is called plasmone.

The relative importance and role of cytoplasm and nucleus in ontogenic development have been subjects of controversy almost, from the time when two main components of the egg-cell were recognized. The controversy, in fact, is a philosophical extension of the problem already existing. The cytoplasm versus nucleus controversy acquire a sharp edge because while it was realized that the nucleus is the repository of all genetic information. In the following account an attempt is made to present evidence of the role and total interdependence of the cytoplasm and nucleus.

Importance and role of cytoplasm in development

Different experiments have shown that the egg cytoplasm plays an important role in early development. Recent experiments on sea urchin indicate that, for cleavage to occur the following two cycles in the cell need to be in harmony: that of nuclear division and that of the protoplasmic activity. The protoplasmic shows cyclic changes in the thickness of hyaline layer and the amount of sulphydryl (-SH) content in the cortex. Heat (34°C) treatment (2.5% in sea water) suppresses the -SH cycle and cleavage (cytokinesis) but allows the nuclear division to continue. The UV irradiation of egg, on other hand suppresses the nuclear division cycle and cleavage but allows the -SH cycle to take place. The control of biological clock maintain by the -SH cycles in the cortex and the nuclear division cycles remain to be investigated.

The other example is the grey crescent in amphibians has an important role. Egg cortex seems to be important in the mechanism of cytokinesis. The ring of the microfilaments in the egg cortex is ascribed and instrumental role in the contractile ring theory of cell cleavage. The cytoplasm plays such a major role because it is the store house of not only the yolk but also of genetic information. The eggs of animals typically store large quantities of organic molecules during oogenesis. Among

these "cytoplasmic factors" are proteins and mRNAs that function during early development of the embryo. Both the stored proteins and new proteins made from stored mRNAs can be utilized to control cell functions during cleavage. In most animal embryos (mammals are an exception), these maternal products control development until the blastula or early gastrula stage. Because cell division is so rapid during the cleavage period of development, there is no time for transcription to occur and the embryonic genome is not expressed. Due to the presence of stored cytoplasmic factors, the zygote of some animals can develop to the blastula stage, even if its nucleus is removed. Cytoplasmic factors are not stored evenly within the egg cytoplasm. This is the way that daughter cells initially become different from one another during early cleavages of the embryo. The uneven distribution of stored components is obvious in eggs with large amounts of yolk, such as the amphibian egg.

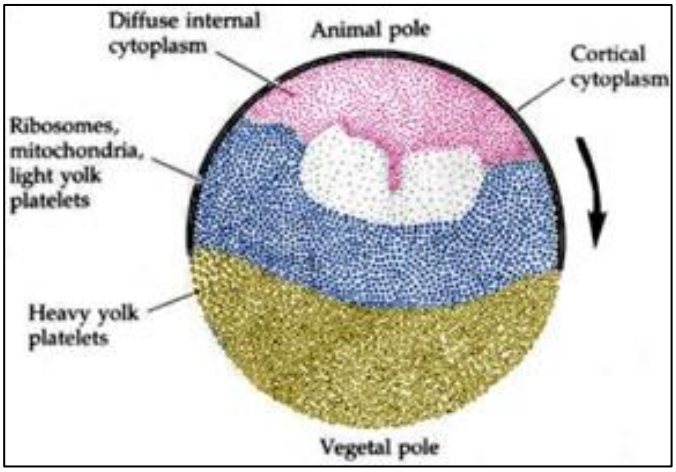

Fig 1: Distribution of egg cytoplasm

Note the unequal distribution of yolk and organelles within this frog egg (Fig 1). The outer "cortical" layer of cytoplasm also differs from the underlying cytoplasm. When a sperm penetrates the egg, the cortical cytoplasm shifts, as shown by the arrow, revealing a grey region beneath.

Experiment 1

One of the earliest experiments to demonstrate the importance of cytoplasmic factors was performed by Hans Spemann in the early 1900's. He used frog eggs, in which the outer layer of cytoplasm shifts by 30° at fertilization to reveal an underlying region, the grey crescent. The following experiment demonstrated the importance of cytoplasmic factors within the grey crescent region.

Spemann noted that the zygote usually divided so that the cleavage plane bisected the grey crescent. If the resulting cells were separated, both cells developed into a normal embryo. Occasionally, the first cleavage occurred in a plane whereby one daughter cell received all of the grey crescent and the other cell received none. In this case, separation of the two cells resulted in a normal embryo from the grey crescent-containing cell, but only a mass of cells from the other daughter cell.

Spemann used a loop of hair to separate the two cells during the first cleavage division as shown in fig 2. We now know that the grey crescent region contains cytoplasmic factors that initiate blastopore formation. Additionally, this region of cytoplasm forms the notochord (after moving inside the embryo during gastrulation) and is responsible for induction of the central nervous system.

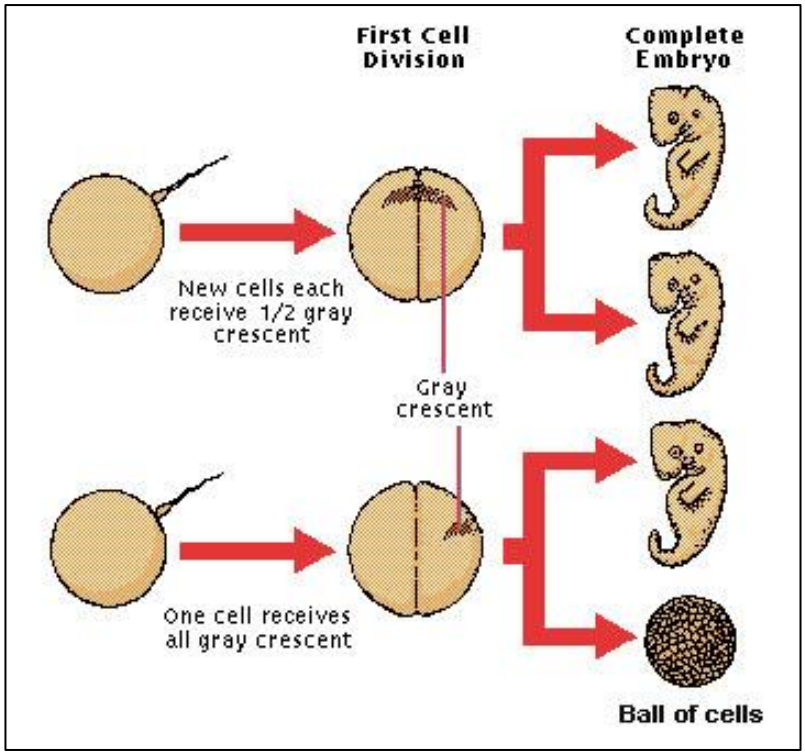

Fig 2: Experiment to demonstrate importance of cytoplasmic factors

Experiment 2

Experiments that inhibit transcription versus translation indicate that the pre-packaged cytoplasmic factors include mRNAs. Inhibition of transcription does not prevent cleavage, whereas inhibition of translation does stop cleavage and thus prevents further development. We now know that several different classes of mRNAs are stored in egg cytoplasm during oogenesis. They are involved in early development by giving rise to proteins that can act in various ways, such as providing signals for induction.

The cytoplasm provides most of the energy for cell through oxidative phosphorylation and anaerobic glycolysis. Cytoplasmic ribosomes are the sites of most translational procedure. Furthermore no molecular signals can reach the nucleus from outside the cell without the conductive activity of cytoplasm.

Biochemical evidence for functional state of genome (role of nucleus)

Cell theory changed the conception of embryonic development and heredity

The cell theory developed between 1820 and 1880 by, among others, the German botanist Matthias Schleiden and the physiologist Theodor Schwann, was one of the most illuminating advances in biology, and had an enormous impact. It was at last recognized that all living organisms consist of cells, which are the basic units of life, and which arise only by division from other cells. Multicellular organisms such as animals and plants could then be viewed as communities of cells. Development could not therefore be based on preformation but must be epigenetic, because during development many new cells are generated by division from the egg, and new types of cell are formed.

A crucial step forward in understanding development was the recognition in the 1840s that the egg itself is but a single, albeit specialized, cell. An important advance was the proposal by the 19th

century German biologist August Weismann that the offspring does not inherit its characteristics from the body (the soma) of the parent but only from the germ cells-egg and sperm and that the germ cells are not influenced by the body that bears them. Weismann thus drew a fundamental distinction between germ cells and somatic cells or body cells (Fig 3). Characteristics acquired by the body during an animal's life cannot be transmitted to the germ cells. As far as heredity is concerned, the body is merely a carrier of germ cells. As the English novelist and essayist Samuel Butler put it: "A hen is only an egg's way of making another egg".

Work on sea urchin eggs showed that after fertilization the egg contains two nuclei, which eventually fuse; one of these nuclei belongs to the egg while the other comes from the sperm. Fertilization therefore results in an egg carrying a nucleus with contributions from both parents, and it was concluded that the cell nucleus must contain the physical basis of heredity.

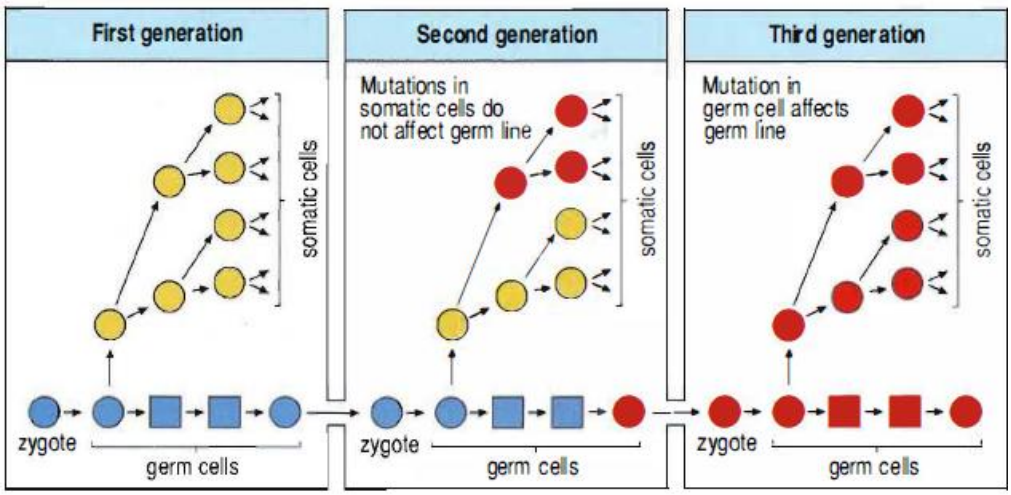

Fig. 3: The distinction between germ cells and somatic cells. In each generation germ cells give rise to both somatic cells and germ cells, but inheritance is through the germ cells only. Changes that occur due to mutation in somatic cells can be passed on to their daughter cells but do not affect the germ line.

The climax of this line of research was the eventual demonstration, toward the end of the 19th century, that the chromosomes within the nucleus of the zygote (the fertilized egg) are derived in equal numbers from the two parental nuclei, and the recognition that this provided a physical basis for the transmission of genetic characters according to laws developed by the Austrian botanist and monk Gregor Mendel. The constancy of chromosome number from generation to generation in somatic cells was found to be maintained by reduction division (meiosis) in germ cells. The diploid precursors to the germ cells contain two copies of each chromosome, one maternal and one paternal. This number is halved by meiosis during formation of the gametes, so that each haploid germ cell contains only one copy of each chromosome. The diploid number is restored at fertilization.

Mosaic and regulative development

Once it was recognized that the cells of the embryo arose by cell division from the zygote, the question of how cells became different from one another emerged. With the increasing emphasis on the role of the nucleus, in the 1880s Weismann put forward a model of development in which the nucleus of the zygote contained a number of special factors or determinants (Fig 4).

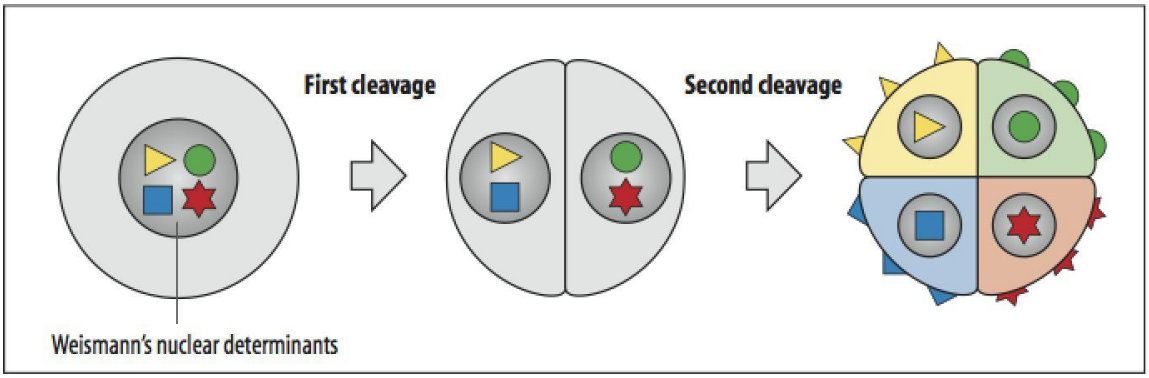

Fig 4: Weismann's theory of nuclear determination. Weismann assumed that there were factors in the nucleus that were distributed asymmetrically to daughter cells during cleavage and directed their future development.

He proposed that as the fertilized egg underwent division (cleavage), these determinants would be distributed unequally to the daughter cells and so would control the cells' future development. The fate of each cell was therefore predetermined in the egg by the factors it would receive during cleavage. This type of model was termed 'mosaic', as the egg could be considered to be a mosaic of discrete localized determinants. Central to Weismann's theory was the assumption that early cell divisions must be asymmetric divisions, so that the daughter cells would be quite different from each other as a result of unequal distribution of nuclear components.

In the late 1880s, initial support for Weismann's ideas came from experiments carried out independently by the German embryologist Wilhelm Roux, who experimented with frog embryos. Having allowed the first cleavage of a fertilized frog egg, Roux destroyed one of the two cells with a hot needle and found that the remaining cell developed into a well-formed half-larva (Fig 5). He concluded that the "development of the frog is based on a mosaic mechanism, the cells having their character and fate determined at each cleavage". But when Roux's fellow countryman, Hans Driesch, repeated the experiment on sea urchin eggs, he obtained quite a different result (Fig 6). He wrote later: "But things turned out as they were bound to do and not as I expected: there was, typically, a whole gastrula on my dish the next morning, differing only by its small size from a normal one; and this small but whole gastrula developed into a whole and typical larva."

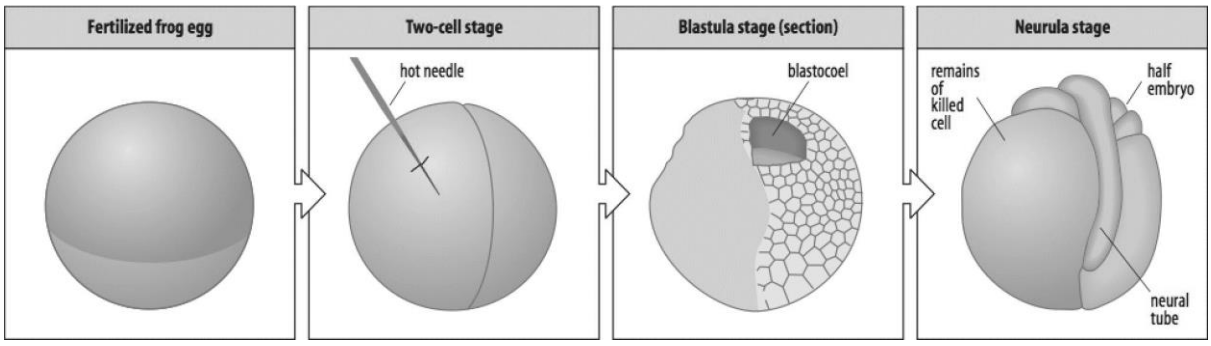

Fig 5: Roux's experiment to investigate Weismann's theory of mosaic development. After the first cleavage of a frog embryo, one of the two cells is killed by pricking it with a hot needle; the other remains undamaged. At the blastula stage the undamaged cell can be seen to have divided as normal into many cells that fill half of the embryo. The development of the blastocoel is also restricted to the undamaged half. I n the damaged half of the embryo, no cells appear to have formed. At the neurula stage, the undamaged cell has developed into something resembling half a normal embryo

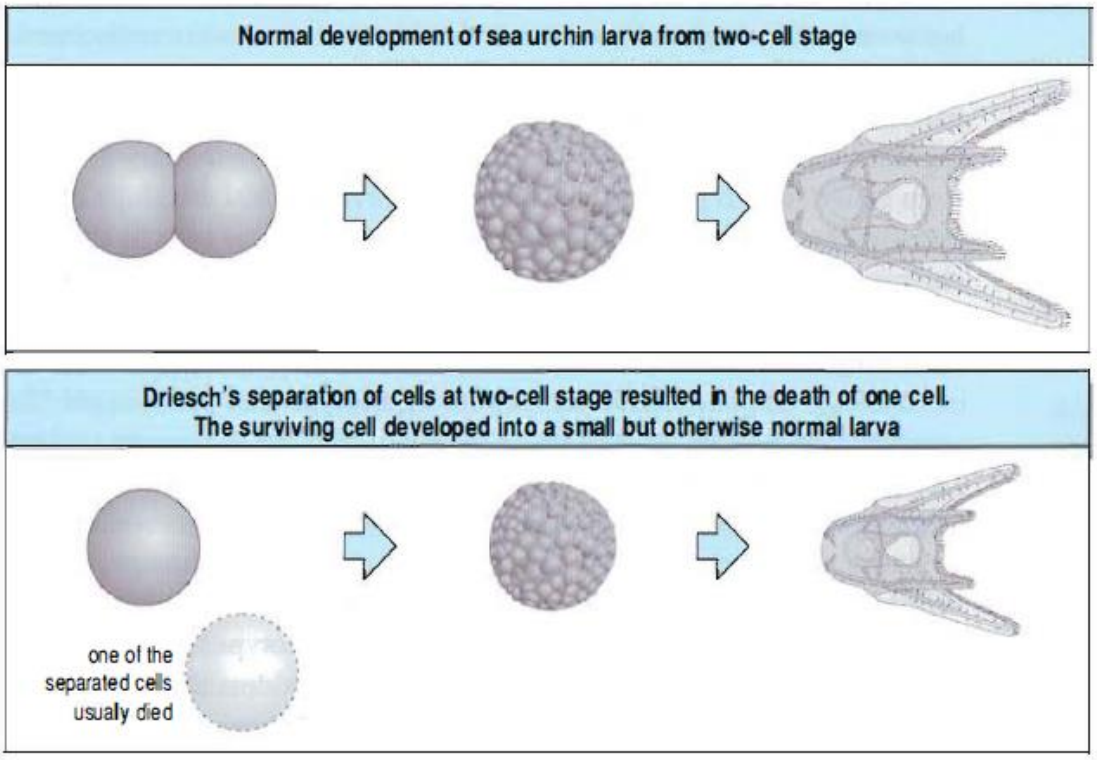

Fig 6: The outcome of Driesch's experiment on sea urchin embryos, which first demonstrated the phenomenon of regulation. After separation of cells at the two-cell stage, the remaining cell develops into a small, but whole, normal larva. This contradicts Roux's earlier finding that if one of the cells of a two-cell frog embryo is damaged, the remaining cell develops into a half-embryo only (see Fig 5)

Inductive interactions can make cells different from each other

Making cells different from one another is central to development. There are numerous examples in development where a signal from one group of cells influences the development of an adjacent group of cells. This is known as induction, and the classic example is the action of the Spemann organizer in amphibians. Inducing signals may be propagated over several or even many cells, or be highly localized. The inducing signal from the amphibian organizer affects many cells, whereas other inducing signals may pass from one cell to its immediate neighbour. There are three main ways in which inducing signals may be passed between cells (Fig 7).

First, the signal is transmitted through the extracellular space, usually by means of a secreted diffusible molecule. Second, cells may interact directly with each other by means of molecules located on their surface. In both these cases, the signal is generally received by receptor proteins in the cell membrane and is subsequently relayed through intracellular signalling systems to produce the eventual cellular response. Third, the signal may pass from cell to cell directly through gap junctions.

These are specialized protein pores in the apposed plasma membranes, which provide direct channels of communication between the cytoplasm of adjacent cells through which small molecules can pass. In the case of signalling by a diffusible molecule or by direct contact, the signal is received at the cell membrane. If it is to alter gene expression in the nucleus, the signal has to be transmitted from the membrane to the cell's interior. This process is known generally as signal transduction, and is carried out by relays of intracellular Signalling molecules that are activated when the extracellular signalling

molecule binds to its receptor. These intracellular signalling proteins and small-molecule 'second messengers' interact with one another to transmit the signal onward in the cell.

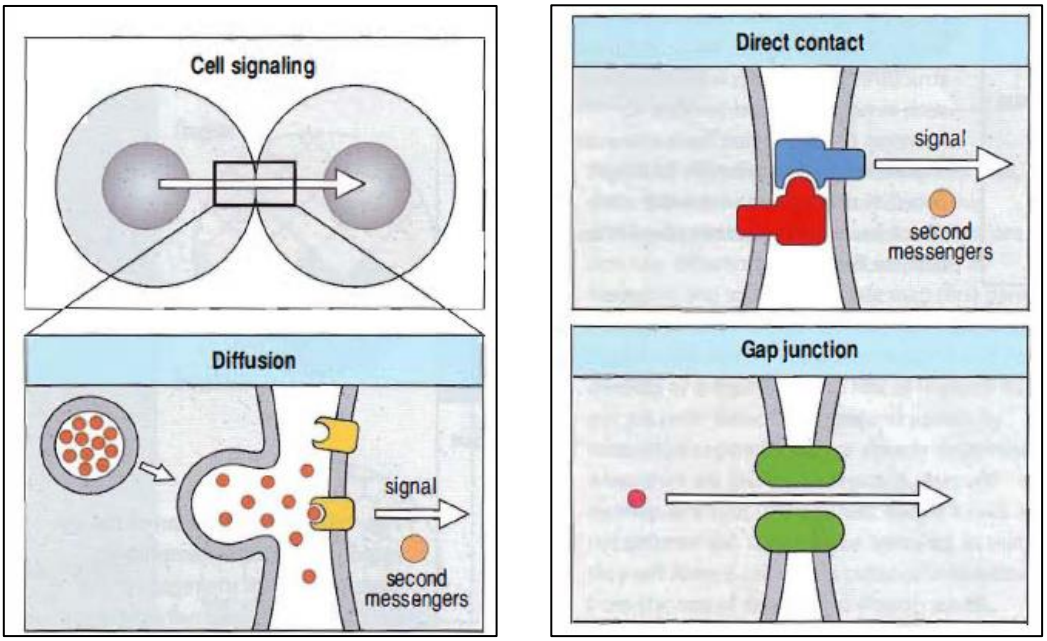

Fig 7: An inducing signal can be transmitted from one cell to another in three main ways. The signal can be a diffusible molecule, which interacts with a receptor on the target cell surface (second panel), or the signal can be produced by direct contact between two complementary proteins at the cell surfaces (third panel). If the signal involves small molecules it may pass directly from cell to cell through gap junctions in the plasma membrane (fourth panel).

Regulation of protein activity by phosphorylation is an important component of most signalling pathways. Different signals received by a cell can be integrated by the interaction of the different signalling pathways. A further important feature of induction is whether or not the responding cell is competent to respond to the inducing signal. This competence may depend on, for example, the presence of the appropriate receptor and transducing mechanism, or on the presence of the particular transcription factors needed for gene activation. A cell's competence for a particular response can change with time; the Spemann organizer can induce changes in the cells it affects only during a restricted time window. In embryos, it seems that small is generally beautiful where signalling and pattern formation are concerned. Whenever a pattern is being specified, the size of the group of cells involved is barely, if ever, greater than 0.5 mm in any direction; that is, some 50 cell diameters. Many patterns are specified on a much smaller scale and involve just tens or a few hundred cells. This means that the inducing signals involved in pattern formation reach over distances of the order of only ten times a cell diameter. The final organism may be very big, but this is almost entirely due to growth of the basic pattern.

The crucial biochemical evidence has accumulated only recently from the DNA-RNA hybridization technique. The technique is quite simple in principle. It is based on the properties of specific base-pairing in the double stranded nucleic acid molecule. This means a particular DNA strand will base pair (or anneal) only with its own mRNA transcript (complementary mRNA) and not with any other mRNA. But this technique is very complicated in practice. The interpretation of data is at time not

easy and leaves doubt. Despite this drawback, nucleic acid hybridization is an extremely powerful tool. Some of the result are given here.

Hybridization results - The hybridization experiment indicate the existence of not more than three distinct abundant classes of mRNA. In each class the number of mRNA copies of each DNA sequence per cell varies and the average sequence concentration differs by more than 10 folds. For example a comparison of three mouse tissues liver, kidney and brain indicates that some mRNA sequences are tissue specific while others are shared by two or more tissues. In other word some genes are transcribed specifically in each tissue while other genes are transcribed in common with other tissue which means that genome in the three tissues is in different functional states.

Nature of changes in nuclei

The question whether nuclei undergo Irreversible changes during development has engaged the attention of developmental biologists. Answer is neither yes nor no. In very few animals, like *Ascaris*, the chromosomes undergoes diminution in the somatic cell. This changes of nucleus are irreversible. On the other hand, in the germ line cell it is necessary to retain the entire gene compliment to maintain constancy of the species characters. The germ line nucleus therefore cannot undergo irreversible changes. It is possible that during the development of somatic cell, on the other hand, some nuclei undergo irreversible changes, some partially reversible changes and other completely reversible changes.

Cell hybridization

Cell fusion (somatic cell hybridization) is the technique of combining two cells from different tissues or species in a cell culture. The cells are fused and coalesce but their nuclei generally remain separate. However, during cell division a single spindle is formed so that each daughter cell has a single nucleus containing sets of chromosomes from each parental line. Subsequent division of the hybrid cells often results in the loss of chromosomes (and therefore genes), so that absence of a gene product in the culture can be related to the loss of a particular chromosome. Thus the technique is used to determine the control of characteristics exerted by specific chromosomes. Hybrid cells (hybridoma) resulting from cell fusion have also been used to produce monoclonal antibodies.

Formation of Somatic Cell Hybrids

It had been known since the 60's that somatic cells from the same or different species in culture could spontaneously fuse to form polyploid cells. The product of fusion was called *homokaryon* if the two parental cells came from the same species, and *heterokaryon* or *somatic cell hybrid* if the fusion was interspecific. The hybrid cells could divide by mitosis and proliferate and thus could be maintained in culture (Fig 8).

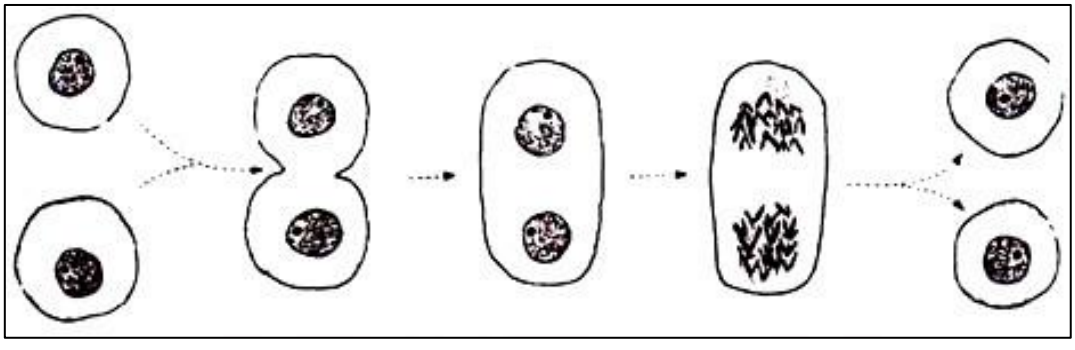

Fig 8: Mechanism of formation of somatic cell hybrids

Two further technical advances made human gene mapping by somatic cell hybrids possible. In 1962 Okada discovered that inactivated Sendai virus could greatly increase the rate of cell fusions. Since then several agents causing cell fusion have been tried among which polyethylene glycol has some advantages. The exact mechanism of cell fusion is not known.

In the case of UV-inactivated Sendai virus, it seems that the virus absorbs to the cell surface leading to agglutination of cells. The protein coat of the virus forms the connecting bridge between the cells.

The membranes of the two cells swell into this region and when they come in contact are dissolved. The cell contents mix up, the nuclei fuse and a heterokaryon is formed. When cell fusion is mediated by polyethylene glycol, the two cell membranes directly come in contact.

The second technical advancement was the finding that when sub-lines of hybrid cells are maintained in culture, there is gradual and preferential loss or retention of specific chromosomes. The association between the retention of a genetic marker and that of a specific human chromosome could be determined.

In mouse-man hybrids, most of the chromosomes of the mouse are retained. By using a selective medium which allows growth of cells having a particular chromosome, it is possible to locate genes on a specific chromosome. This technique has been extensively applied for human gene mapping.

The following example illustrates the technique used by Littlefield (1964) for assigning genes to specific chromosomes. He used deletions and mutations affecting enzymes involved in the purine and pyrimidine salvage pathways, namely thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT). There are two pathways for DNA synthesis.

In the first, which proceeds under normal conditions, DNA is synthesised from simple organic molecules and the necessary enzymes (De novo pathway). The second is the alternate or salvage pathway which utilizes nucleotide precursors for DNA synthesis (Fig 9)

The salvage pathway is followed only if the first pathway is blocked by an antimetabolite (for example aminopterin) or by a mutation. Two enzymes are necessary for the salvage pathway, HGPRT and TK. If even one of the two enzymes is absent, DNA synthesis cannot take place by the salvage pathway.

Cells from a mutant mouse cell line deficient in the enzyme TK (i.e. TK–/HGPRT+) were mixed with cells from a human line deficient in HGPRT (i.e., TK+/HGPRT–) and allowed to grow on minimal medium. Under appropriate conditions the cells fused to form hybrid cells KT+/TK–; HGPRT+/HGPRT–). It should be noted that in the hybrid cells there is one normal allele for the enzyme TK (from the human cell line) and one normal allele for HGPRT (from the mouse cell line).

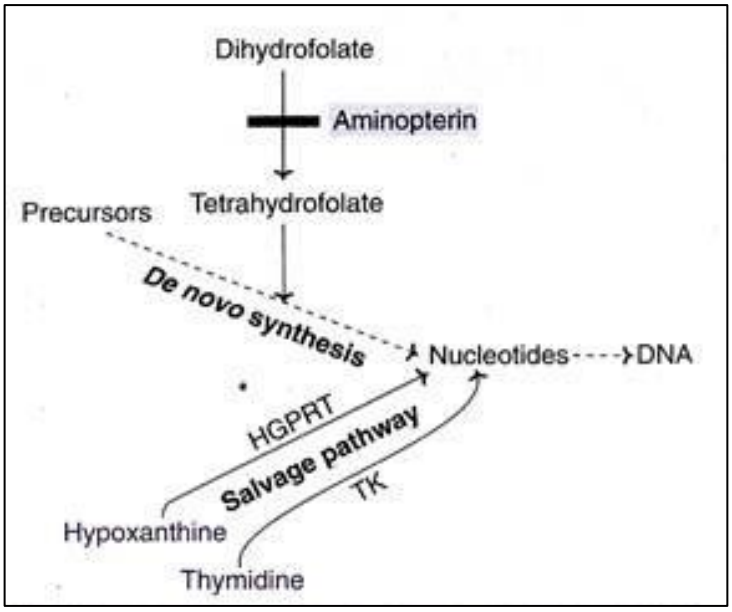

Fig 9: Pathways for the synthesis of nucleotides (HGPRT and TK)

All the cell lines could grow on a minimal medium. The mouse cell line (TK–) is not able to grow on a medium containing thymidine as the cells are deficient in TK. Similarly the human cell line (HGPRT–) cannot grow on a medium containing hypoxanthine due to lack of the enzyme HGPRT.

It is also noteworthy that neither of the mutant cell lines is able to grow if aminopterin, an antimetabolite is present in the medium. Aminopterin acts by inhibiting the enzyme folic acid reductase which catalyses the synthesis of reduced folate. The latter is required in the various steps of the normal pathway leading to the synthesis of DNA.

In the presence of aminopterin therefore, DNA is synthesised through the salvage pathway, but only if the enzymes TK and HGPRT are available. The mouse-man hybrid cells are thus able to grow in presence of aminopterin if thymidine and hypoxanthine are present in the medium.

There is a selective medium which allows growth of the hybrid cells but inhibits the parental cells. This medium contains hypoxanthine, aminopterin and thymidine and is called HAT medium. The hybrid cells will proliferate on HAT medium to form colonies as they alone have genes for both TK and HGPRT. Colonies of hybrid cells can be sub-cultured and cloned for mapping genes.

Sub-lines which show progressive loss of human chromosomes are maintained. Only those cells that retain the specific chromosome having the gene for thymidine kinase would survive in HAT medium. It was found out that only cells retaining chromosome 17 could grow on HAT medium. Obviously the gene for thymidine kinase is located on chromosome 17.

The mouse-man cell hybrids have the following advantages for studies on gene mapping:

1. Preferential loss of human chromosomes.

2. Availability of cell lines with identifiable human phenotypes different from those in rodents.

3. The apparent distinction between rodent and human chromosomes in interspecific hybrids.

4. That both rodent and human genes are simultaneously expressed in the cell hybrids and the product proteins of each can be identified individually.

5. The linkage groups on mouse chromosomes are known.

Nuclear transplantation experiments

Nuclear transplantation is a method in which the nucleus of a donor cell is relocated to a target cell that has had its nucleus removed (enucleated). Nuclear transplantation has allowed experimental embryologists to manipulate the development of an organism and to study the potential of the nucleus to direct development. Nuclear transplantation, as it was first called, was later referred to as somatic nuclear transfer or cloning.

Yves Delage first wrote about nuclear transplantation in 1895, speculating that if one were to replace an egg nucleus with another egg's nucleus, full development would occur. Later in 1938, Hans Spemann suggested an experiment whereby, using technologies not yet available to him, one could remove the nucleus of an egg and replace it with a different nucleus extracted from a developed cell. Thomas King and Robert Briggs were the first to perform experimental nuclear transplantation. The technique was soon after used by John Gurdon and eventually led to the first clone of a mammal, "Dolly" the sheep, by Ian Wilmut in 1996.

Nearly fifteen years after Spemann wrote about the possibility of nuclear transplantation, Briggs and King, using northern leopard frogs (*Rana pipiens*), performed the first nuclear transplantation experiment. They transplanted the nucleus from an early stage embryo to an unfertilized egg that had been enucleated. The egg cell was pricked with a clean glass needle in order to induce a fertilization-like response. The faux activation of fertilization allowed for extraction of the nuclear material inside while also activating the host egg cell. Meanwhile, the nucleus of a donor cell was extracted and then inserted into the newly enucleated and activated egg cell. That process induced development of the host egg according to the instructions of the newly inserted nucleus, resulting in the formation of an organism with the same genetic material as the donor cell, or a clone.

Briggs and King continued to examine the potential of differentiated cells throughout the 1950s. They found that if the donor nucleus was extracted later in development, the potential of directing full development in the activated egg cell was greatly reduced. After the Briggs and King experiments it was generally accepted that the nuclear material in developing cells slowly loses its potential for full development.

That view was challenged in 1958 when Gurdon's experiments with African claws frogs (*Xenopus laevis*) produced fully developed frogs from the transferred nucleus of cells much later in development. Gurdon allowed the cloned frogs to develop to sexual maturity and was then able to mate two sexually mature clones, suggesting that the donor nuclei were able to fully redirect development. Gurdon's experiments were widely accepted by the scientific community but questions remained for several decades. Scientists were concerned about whether the nucleus of the host egg cell was truly enucleated. The question of whether remnants of the host egg cell or the inserted nucleus directed development remained unanswered from 1958 to 2002, despite many attempts by Gurdon to prove it was the inserted nucleus.

In 2002, however, Konrad Hochedlinger and Rudolf Jaenisch published an experiment using nuclear transplantation of mature white blood cells to generate mouse clones. Hochedlinger and Jaenisch were able to show that the inserted nucleus induced development in the host egg cell.

Although experimental embryologists continued to use nuclear transplantation to create clones of several species, Ian Wilmut's cloning experiment in 1996 was a controversial and widely publicized cloning experiment. Dolly was cloned using the nucleus of a mammary gland cell from an adult sheep and transplanting it into an enucleated egg cell from another sheep. The activated egg cell was then transferred into a third surrogate sheep that carried Dolly to term. Dolly died at the age of to six due to

lung disease and severe arthritis, and although her death was not attributed to the fact that she was a clone, many believe that the relationship between telomeres and aging was the reason for her demise.

Nuclear transplantation may have begun as a subtle idea in the late 19th and early 20th centuries, but it evolved into a feasible and widely used process by experimental embryologists in the late 1990s. The cloning of Dolly the sheep worried many about the possibility of human cloning and the moral boundaries of modern advances in science. In the context of the embryonic stem cell discourse of the late 1990s and early twenty-first century, somatic nuclear transfer has been contrived into moral arguments about rights of the human embryo. Furthermore, nuclear transplantation has spurred ethical discussion on the value of a human life during all stages of development. Many scientists have abandoned the methods involved in nuclear transplantation and have adopted methods set forth by Shinya Yamanaka in his experiments involving induced pluripotent stem cells.

Role of nucleus in RNA and protein metabolism:

- A couple of days after the enucleation the ergastoplasmic material decrease in its amount.
- Electron microscope study suggests removal of nucleus does not effect on the structure of mitochondria.
- Quantitative estimation of RNA shows that there is a marked decrease in RNA in enucleated halves file in nucleated halves it remain constant.
- Thus it can be said that nucleus control the amount of RNA in cytoplasm and thus in absence of nucleus RNA concentration fall in cytoplasm. There since that synthesises RNA that transferred to cytoplasm as mRNA. But cytoplasm does not inactive in this respect because small amount of RNA may also synthesise in enucleated halves so nucleus is not the only site for protein synthesis in *Amoeba*.

Methodology

The actual techniques in SCNT (Somatic Cell Nuclear Transfer) are common between different researchers, however they differ by the materials and equipment used, for example, specific growth chemicals and electric fusion voltages. Apart from this, they all are common centrally - they involve the nucleus of a somatic cell (e.g. a normal body cell such as a blood cell, heart cell/cardiocyte, skin cell/fibroblast; the sperm and egg are germ cells not somatic cells) being physically transferred into an unfertilised egg cell that has had its own nucleus removed (referred to as 'enucleation').

Stated below is an outline of the steps you would take in order to perform SCNT for humans.

Step 1: Preparation of the somatic cell

The somatic cell, as stated, can be any type of normal cell in the body apart from the sperm or egg. Most researchers appear to favour skin fibroblasts, because the skin is easy to access, non-invasive and fairly painless. However, cells from the breast/mammary gland and cumulus cells have also been used.

A tiny amount of skin is cut and placed in a trypsin enzyme-buffer solution that frees the target fibroblasts from the extracellular matrix. The mixture is placed on a serum medium and incubated for three weeks, in order to obtain a single layer of fibroblasts without any other cell types.

Step 2: Preparation of the egg/oocyte

Typically, researchers will select the target egg that is in the antral stage and exhibits the 1st polar body. When the researchers can see follicles at least 18mm wide, human chorionic gonadotropin

(hCG) is injected into the female donor. hCG is used since it is a strong inducer of ovulation, and allows a more 'comfortable' way of obtaining the egg without any invasive and direct surgical procedure to the ovaries and the donors themselves. Consequently, the ovulated egg is collected by ultrasound-guided transvaginal needle aspiration in a procedure similar to in-vitro fertilisation.

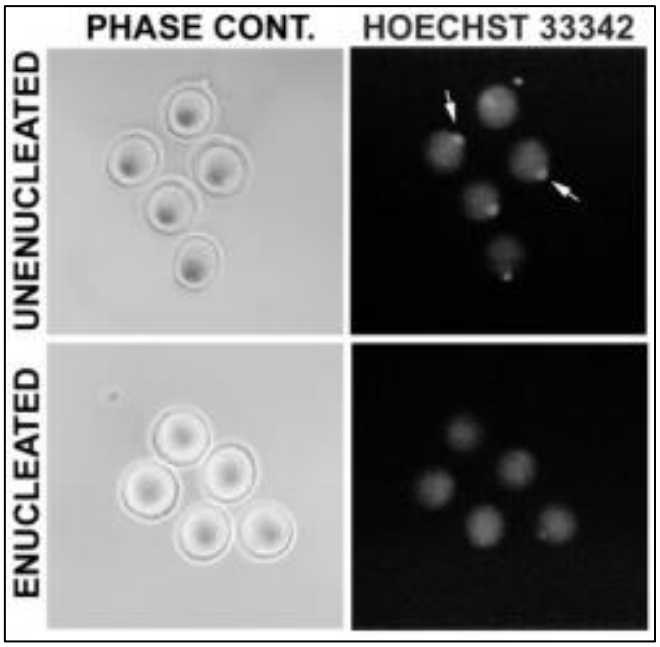

Fig 10: Step 2 - Generating cytoplasts by oocyte enucleation

Once the egg is extracted, it is placed in liquid human serum. Fluorescent tags are bound to the oocyte's DNA, allowing the researchers to check all the oocyte's DNA/nucleus has been removed when exposing the egg to UV light.

The egg's nucleus is removed using an inverted microscope, UV light and a glass needle. This setup minimises damage to the delicate egg as it can cut through the thick zona pellucida shell, and is fairly easy to manipulate. At this point with its nucleus removed, the oocyte is called a cytoplast (Fig 10).

Step 3: Nuclear Transfer

Both fibroblast and egg are placed in a thin human serum solution with cytochalasin B.

Once the donor fibroblast's nucleus is extracted from the fibroblast with a pipette, it is called a karyoplast. Subsequently, this karyoplast is injected into the egg/cytoplast past the zona pellucida (Fig 11).

At this point, the karyoplast and cytoplast are still functionally separate (see Fig 12 at 0 minutes), therefore a few electric pulses are given to the entire solution causing fusion between the two entities (see Fig 12 at 10 minutes).

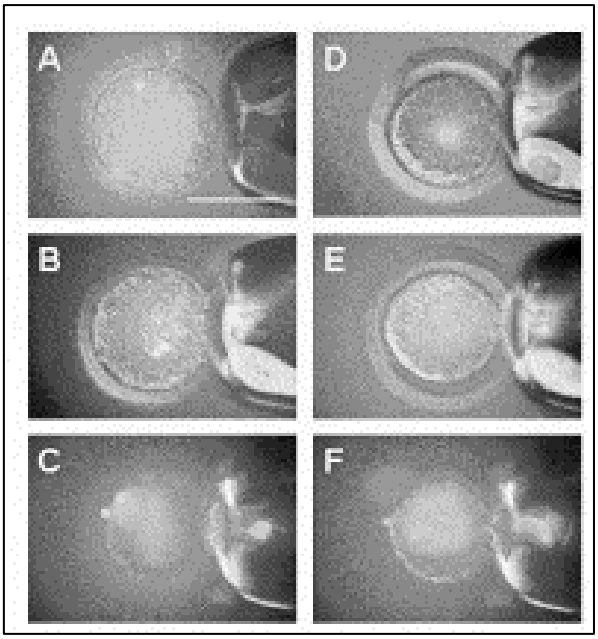

Figure 11: Step 3 - An adult cell nucleus is injected into an enucleated egg.

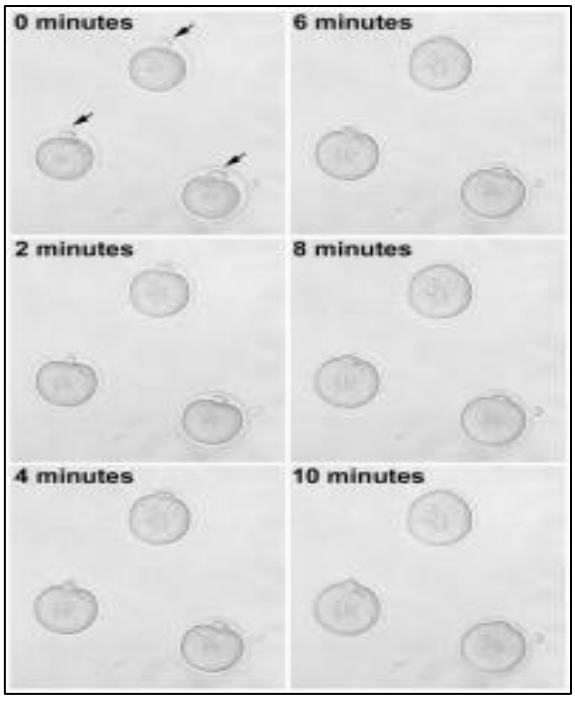

Fig 12: Step 3/4 - Time-lapse images of fusion between fibroblast (arrowed) and cytoplast/egg

Step 4: Post Nuclear Transfer Procedures

The complete process of nuclear transfer is completed approximately 35-45 hours after the original hCG was administered to the female donor. However, it takes an additional three hours before cleavage can be seen if the transfer and activation has been successful.

Finally, the egg is incubated in a culture medium at 37°C in highly humidified conditions. This could be both an artificial attempt and natural requirement that replicates the uterine conditions, which are

conducive to embryonic development. After this activation, in approximately four days for human donors, cleavage of the egg can be clearly seen.

It has been noted by Dominko and colleagues that the oocyte can be from any mammal if using a mammalian nucleus. It does not necessarily mean a human fibroblast must require a human egg for use. This is, because the initial development of all mammal eggs undergo a similar process, and it is only later in the morphogenesis of the embryo that the nucleus's DNA actually starts taking control of the process. The consequence of using a cow egg with a human fibroblast or other species' fibroblast is the first 2 cleavages correlated to bovine development time, while after these two divisions, the growth rate and timing of the embryo matched the donor species. Therefore, if human nucleus and oocyte were used, the entire process would mirror the normal human rate.

Step 5: Embryogenesis

Once the hybrid egg has developed into a blastocyst, what happens to it from this point depends on its application:

- For reproductive cloning (creating an entire organism): the blastocyst is implanted into a surrogate mother who carries the developing embryo like a normal pregnancy.
- For applications in regenerative medicine (obtaining specific cell/tissue types that can be surgically grafted for a patient): the ICM is harvested from the blastocyst onto mice-derived feeder cells for nutrients and differentiated into the required tissue/cell types, using certain growth and differentiation factors over two days.

The actual differentiation factors required for specific somatic cells has been determined over the years by many different researchers, for example, stem cells exposed to dimethyl sulfoxide would diffentiate into different proportions of muscle cells, while stem cells exposed to retinoic acid would become neurons

Apart from the differentiation factors, the removal of the feeder cells or the cells' chemical messengers (cytokines) would also be required to signal the embryonic stem cells to differentiate.

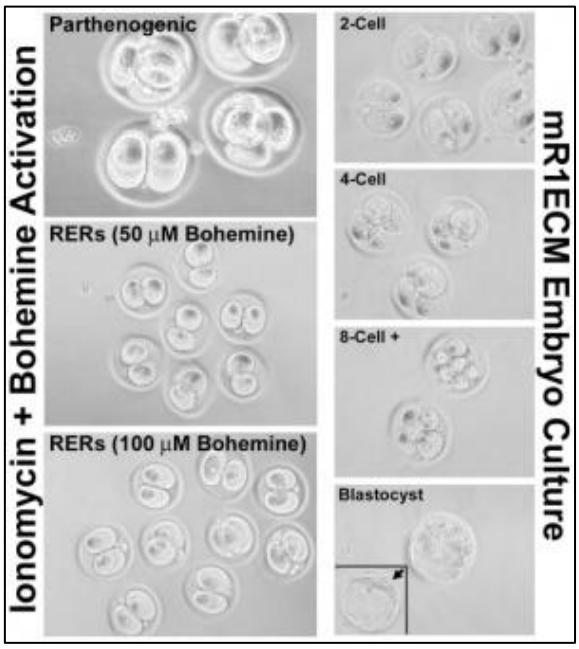

Fig 13: Step 5 - Time-lapse images of embryo development of blastocyst (arrowed) after activation

Current Situation

Unfortunately, current research has not been successful in producing human embryos that develop the blastocoel which is essential for later gastrulation and morphogenesis. It appears the cells remain alive and viable as the cell numbers keep increasing, but they fail to organise themselves into any identifiable embryonic structures. In other primates such as monkeys the production of embryonic stem cells has also been unsuccessful - apparently as a result of large amounts of genetic instability.

There is a high rate of blastocysts that are transplanted into surrogate mothers that result in none reaching full term. Instead, the fallopian tubes, where the embryo was transplanted, develop large sacs of fluid surrounding the dead blastocyst.

Due to ethical issues, the transplantation of human blastocysts in reproductive cloning has not occurred to date. However, blastocyst transplants of other animals such as cows have been successful in producing undamaged and living offspring.

Applications of SCNT

Veterinary, Animal Science

- **Mass production of animals**: As farm animals are being used for human use, SCNT can be used to produce high quality farm animals in infinite number. Cloning technology can be applied, without compromising animal welfare, if integrated in breeding programs and these transgenic clones will be delivering the expected products. Researches show that, somatic cell cloned cattle reportedly were physiologically, immunologically, and behaviourally normal and this makes use of SCNT useful for mass production.
- **Conserving wild animals for next generations**: Another area where SCNT can be useful is conservation. This use can be effective to preserve and propagate endangered species that are being produced poorly in the zoos. With effective reproduction, these species can be reintroduced to the wild again, allowing maintenance of genetic diversity of species by introducing new genes. The use of SCNT can also be helpful to even create the extinct species, if any tissues or cells are available. The idea of producing mammoth is being considered as an intact animal was discovered frozen in the tundra. The close relative of the mammoth, the elephant, could be used both as a surrogate mother and an oocyte donor.

As also mentioned by Holt et al, Reproductive cloning, by nuclear transfer, is often regarded as having potential for conserving endangered species. Cloning non-mammalian vertebrates can be more practical than using conventional reproductive methods. As cloning technology has made a good progress in amphibians, it may be possible to breed threatened amphibians and even reproduce extinct amphibian species.

- **Disease resistant animal production**: By using SCNT, genes causing diseases can be manipulated in order to have healthier farm animals that live a lot longer.

Human Medicine

- **Human therapeutic proteins**: Human proteins are needed and in demand for the treatment of diseases. Purifying proteins from blood is an expensive procedure and also carries the risk of contamination by Hepatitis C or HIV. Proteins can be produced in human cell culture but the output is small and it is also an expensive procedure. But also, Ng et al. mentions that human proteins can be produced in the milk of transgenic sheep, goats and cattle. The output can be as high as 40 g per litre of milk and the cost of the procedure is not as high. By using nuclear

transfer, it is possible to insert human genes at specific points in the genome, improving the reliability of their expression; deleting, substituting and adding of genes that are missing in the patient to lead them have better lives, possible as well.

- **Xenotransplantation**: Shortage of organs is a big problem considering the amount of patients needing them. Transplant organs can be a solution for this. Genetically modified animals such as pigs are being developed as a solution but so far the modifications are limited to adding genes. By using SCNT, deleting genes that are responsible for rejection from pigs is possible and this way it is aimed to avoid rejection of an organ transplanted from a normal pig to a human patient.
- **Animal model for diseases**: experimental animals with altered disease-causing genes can be tailor generated using SCNT, allowing better understanding of the complex pathogenesis of the diseases, eg. Good SCNT-mouse models would allow cystic fibrosis between the lung and intestine to be understood better.
- **Cell Therapy using dedifferentiated stem cells**: This use is being developed for a range of diseases including heart attack, stroke and diabetes etc. Patient's own cell can be used as transplanted cells are likely to be rejected. Cloning of adult animals shows that egg and the embryo have the capability of reprogramming. This use may make it possible to reprogramming patient's own cells without creating and destroying embryos. The differentiating stem cells can then be grown into several hundred or thousand cells and surgically transported into the patient where they will produce the required tissue. To give an example, a child's problem of severe immunodeficiency due to chemotherapy and whole body radiation because of having Hodgkin's Lymphoma, can be corrected as child's skin fibroblast can be obtained and embryonic stem cells obtained as per the procedures mentioned above. Transcription factors would differentiate the stem cells into bone marrow cells, which can be transplanted into the marrow cavities of the child, and they would gradually rebuild the child's haemopoietin system and also, their immune system.

Developmental Biology

- Events during fertilization and pre-implantation embryos: knowledge of the complex mechanisms and the various controlling factors during embryonic development will be understood better with SCNT

Probable questions:

1. Describe the role of cytoplasm in development.
2. Elaborate Spemann experiment with diagram.
3. Discuss the biochemical evidence for functional state of nucleus.
4. Describe with diagram the Weismann's theory of nuclear determination.
5. Describe Roux's experiment.
6. Elaborate how inductive interactions make cells different from each other?
7. How somatic cell hybrids are formed?
8. What is the function of aminopterin?
9. Elaborately describe the nuclear transplantation experiment.
10. What are the applications of SCNT?
11. What is Xenotransplantation?

Suggested readings:

1. Carlson, B.M. (2014). Human Embryology and Developmental Biology. 5th Edn. Elsvier.

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7. Gilbert, S. F. (2010). Developmental Biology, IX Edition, Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts, USA

8. Beetschen, Jean-Claude C., and Jean-Louis L. Fischer. "Yves Delage (1854–1920) as a Forerunner of Modern Nuclear Transfer Experiments." International Journal of Developmental Biology 48 (2004): 607–12.

9. Briggs, Robert, and Thomas J. King. "Transplantation of Living Nuclei from Blastula Cells into Enucleated Frogs Eggs." Proceedings of the National Academy of Sciences of the United States of America 38 (1952): 455–63.

Unit II

Biological specificity: Transplantations and rejection

Objective: In this unit you will know about Biological specificity; transplantation and rejection

Introduction

Many developmental biologists are interested in the molecular signals and cellular interactions that induce a group of cells to develop into a particular tissue. To investigate this, scientists can use a classic technique known as transplantation, which involves tissue from a donor embryo being excised and grafted into a host embryo. The technique of transplantation has served an important role in providing a basic understanding of all facets of biology ranging from cancer and evolutionary biology to developmental biology. First employed by early embryologists, transplantation has played a particularly critical role in elucidating virtually every aspect of embryonic development including cell specification, commitment, cell fate determination, embryonic induction, and plasticity.

The technique of embryonic tissue transplantation has had a long and productive history. The widespread use of embryonic tissue transplantation dates back to the nineteenth century when a new generation of embryologists attempted to move beyond anatomical observations and make embryology an experimental rather than a descriptive science.

The transfer could involve placing the tissue into the same (iso-or homotopic) or different (heterotopic) location, and/or positioning the tissue into an embryo of the same (iso-or homochronic) or different (heterochronic) age. Transplants could also be placed in the same orientation or rotated, and even be placed into the same or different species, with each of these varied manipulations addressing different questions. Using tissue transplantation approaches, these early experimental embryologists not only made considerable strides in elucidating causal relationships in development, but their work also led to the important conceptual advances and the articulation of key embryological concepts such as determination, competence and induction.

However, despite the importance of this early work, progress was limited without availability of techniques for decisive host and donor marking and in the absence of the approaches made possible by the knowledge of modem molecular genetics and cell biology. With the advent of tissue-specific genetic markers and the ability to detect and manipulate gene expression, a new wave of findings has emerged from transplantation experiments specifically related to determination and competence, inducing capacity, fate-mapping, plasticity, and the function of key embryonic genes.

Determination and competence of embryonic tissues

Adoption of a particular cell fate is a multistep process during which cells first become specified, which means they will adopt a cell identity that is in accord with a fate map when developing in isolation in a neutral environment, but are still competent to respond to signals and change their fate if moved to a different region of the embryo. The specified cells then become determined, maintaining their fated identity even when placed in a different embryonic environment. While tissue explants have provided information on the state of specification, transplantation has served as an essential technique to study the state of determination of tissues during various stages of development. Determination is inextricably associated with the competence of a tissue, that is, the ability of a tissue to respond to a specific set of inductive signals. If a tissue is not able to respond to inductive signals from the environment (the competence of the tissue), the tissue will retain its state of determination at the time when it was removed from the host. Manipulating the location where the transplanted tissues

are placed reveals the spatial distribution of states of commitment, while altering the time when transplants are made can reveal the temporal aspects of determination and competence of various tissues within an embryo.

i. One of the most widely studied and classic tissues for transplantation experiments is the *neuroectoderm*, beginning with the iconic studies of Spemann and his group showing that presumptive neural ectoderm from an early gastrula amphibian embryo will assume the fate of its host environment while presumptive neural tissue from a late gastrula will retain its neural fate. These experiments have been repeated using molecular markers in a number of different species, and through a series of heterochronic transplants, researchers were able to conclude that the competence for ectodermal tissue to adopt a neural fate steadily declines as donor embryos age and by neural plate stages the neuroectoderm is largely determined to adopt a neural fate, indicating a gradual commitment of dorsal ectodermal cells for neural differentiation.

ii. Transplantation was also employed to address determination at very early stages of development. In zebra fish, the presumptive enveloping layer (EVL) cells are committed to an EVL exclusive fate by the late blastula stage. As evidence for EVL commitment, when the EVL cells are transplanted heterotopically and heterochronically, they follow unconventional migration paths as a way of compensating to end up in the correct location.

iii. Similar studies in *Xenopas laevis* showed that vegetal pole cells become committed to endoderm by the early gastrula stage. The animal pole cells, on the other hand, pass from pluripotency to a labile state of commitment to ectoderm during the blastula stage. Transplantation of dorsal blastomere cell at 16 or 32 cell stage to a more ventral location either resulted in the formation of a secondary axis, or the repositioning of donor cell progenies to a more animal dorsal location, indicating the ability to form future head organizer is established in dorsal equatorial blastomeres at stage 5 or 6.

iv. The formation of the eye lens has served as a classic model for examining the competence of a tissue. Spemann first reported that most ectoderm during gastrulation possesses lens-forming competence, which was revealed after ectoderm from different parts of the embryo was transplanted to the presumptive eye region and formed lens tissue. The result was corroborated by Henry and Grainger in a more extensive and stage-controlled study, when they transplanted various ectodermal tissues either into the lens-forming region of open neural plate stage host embryos or over the newly formed optic vesicle of somewhat later neurula stage embryos. Using unambiguous molecular markers, they showed that most non-neural ectodermal tissues have some lens-forming potential during early gastrula stages, but that this potential becomes restricted to the presumptive lens-forming region and closely adjacent regions throughout neurula stages. After transplanting animal cap ectoderm to the presumptive lens area, they also discovered that this early gastrula ectoderm has minimal lens-forming competence but possesses considerable neural competence. However, as ectoderm is taken from embryos of increasing age, neural competence is lost and competence to form a lens is acquired. A later ectoderm-to-lens transplantation experiment coupled with in situ hybridization of marker genes demonstrated the linkage between the expression of two genes, Otx-2 and Pax-6, and the competence of presumptive lens tissues to respond to lens-inducing signals.

Case study: Spemann-Mangold organizer

We can see one classic example of cascading events in development by looking at the behaviour of cells in a particular area of the embryo (*Xenopus laevis*, or the African clawed frog; Fig 1)—the area that develops from the grey crescent. What happens to the grey crescent we saw in the zygote? Let's trace where the cytoplasm from this area ends up in two later stages: blastula and gastrula.

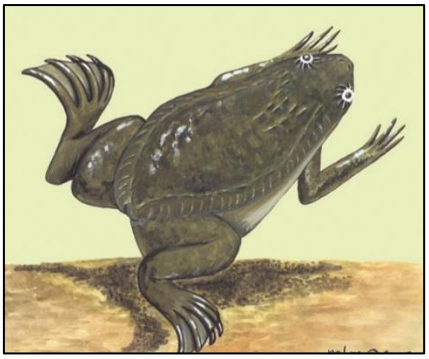


Fig 1: *Xenopus laevis*, or the African clawed frog

The **blastula** is a ball of cells with a hollow space in the middle. In it, the grey crescent cells are found in a group on one side of the embryo, the dorsal side. This is pretty much where the grey crescent was in the zygote.

At the **gastrula** stage, however, these cells do something more interesting: they start marching into the interior of the embryo, causing the tissue to fold inward. The site where the cells migrate into the interior of the embryo is called the *blastopore*, and the grey crescent cells make up its *dorsal lip* (Fig 2).

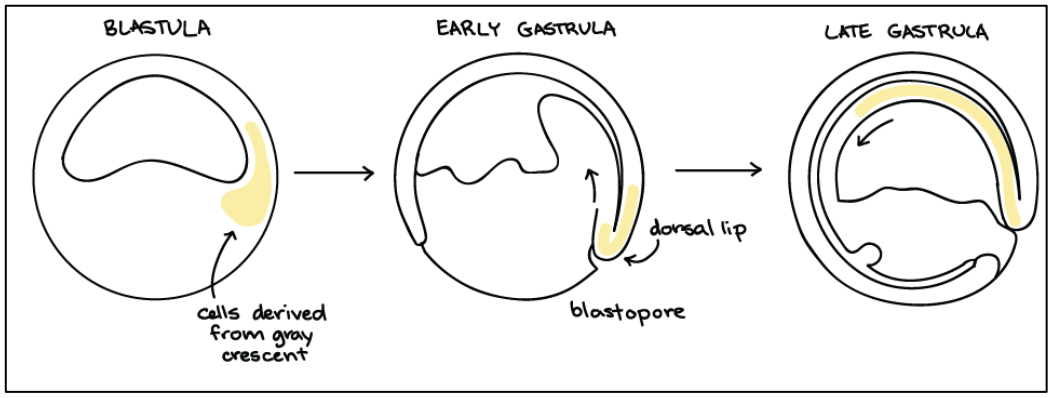
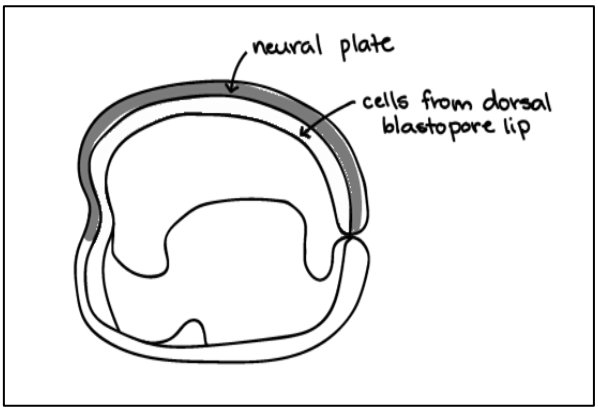


Fig 2: Structure of morula and gastrula

What is the purpose of all this complex cell migration? For one thing, it's key to forming multiple layers of tissue in the embryo. But it's not just a matter of creating more layers; it's also a matter of cells in different tissues "talking" with one another and, in some cases, changing each other's fate. For instance, we now know that the cells that migrate inward instruct the cells above them, a type of tissue called *ectoderm*, to develop into neural—nervous system—tissue.



This interaction was first discovered in the 1920s by Hans Spemann and Hilde Mangold, in what is now one of the most classic experiments in embryology. Spemann and Mangold took the dorsal blastopore lip from a light-coloured newt embryo and transplanted it into the belly, or ventral, side of a dark-coloured newt embryo. This was a very technically demanding experiment, and Mangold worked for years to get five embryos in which it worked correctly

Normally, the tissue at the transplant site would have turned into skin on the newt's belly, the ventral side. However, when the chunk of dorsal blastopore lip was transplanted in, its cells migrated inward, creating a second, functional gastrulation site opposite to the normal one. A new neural plate—the precursor of the spinal cord and brain—appeared at this second gastrulation site. In the end, an entire second newt formed from the belly of the original! (Fig 3)

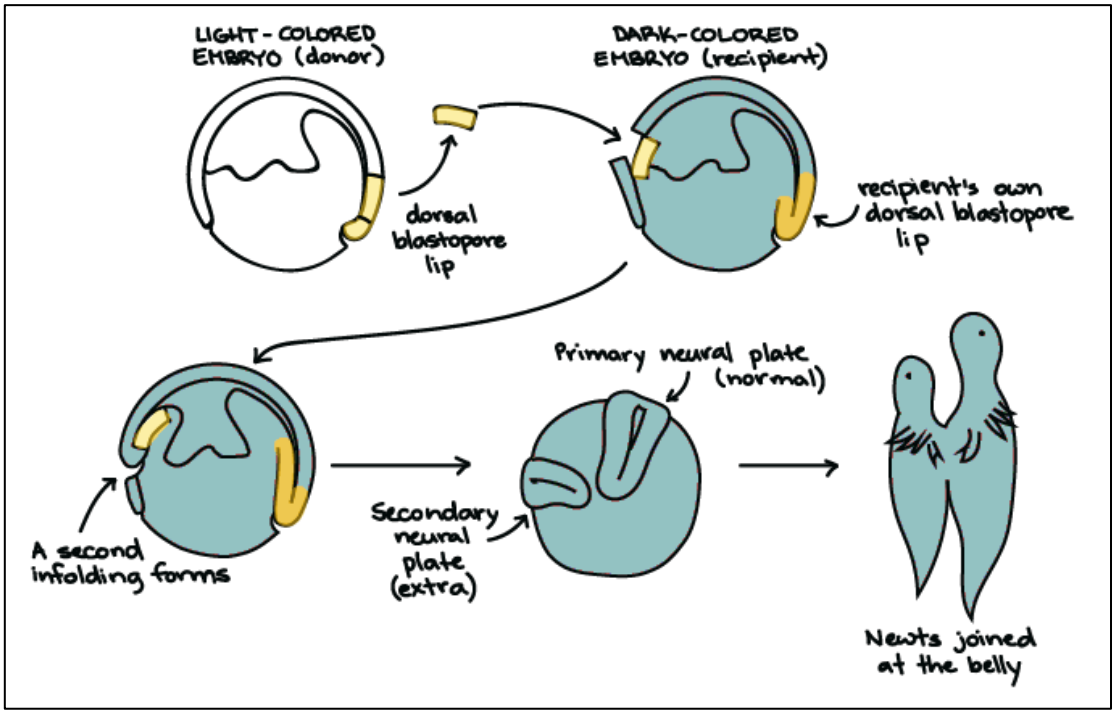


Fig 3: Diagrammatic view of Spemann experiment

What exactly happened in this experiment? There were two basic possibilities for how the transplanted tissue could have led to the formation of the second newt:

- The transplanted tissue might have developed into the second newt all by itself, building its structures from the small group of transplanted cells.
- The transplanted tissue might have "talked" to the layers of recipient tissue around it, organizing their behaviour so that they—along with the transplanted cells—coordinated to form a second newt.

Thanks to the use of different-coloured newts as donor and recipient, Mangold and Spemann were able to tell which possibility was correct. The structures found in the second newt's body consisted of some donor—light—cells but mostly recipient—dark—cells, meaning that cells in the transplanted tissue must have “talked” with the recipient cells nearby and induced them to change their behaviour. This is a classic example of *induction*, in which a cell or tissue communicates with neighbouring cells or tissues to alter their development.

Today, the cells of the dorsal blastopore lip and their descendants are called the *Spemann-Mangold organizer*. Two of the organizer's key roles are to specify dorsal—back, rather than belly—fate and to make nearby ectoderm turn into neural tissue. However, the organizer also guides head-tail axis development and other processes.

Importantly, the organizer itself doesn't directly guide the development of the entire newt. That is, it doesn't pull the strings, so to speak, that make each neuron in the newt's brain or photoreceptor in the newt's eye develop. Instead, it starts a chain reaction of molecular induction events that lead, domino-like, to the formation of the many complex structures of the newt's body—or, in the case of a transplant, to a second newt body!

The organizer acts largely by releasing secreted proteins that diffuse into the surrounding tissues and affect their behaviour. For instance, some of the proteins released by the organizer bind to and neutralize *other* secreted proteins, which instruct cells to develop as skin. By interfering with the "Develop as skin!" cues, the organizer signals allow the overlying tissue to develop as neural tissue, actually its default path.

Inductive ability of embryonic tissue

While the competence of embryonic tissue is extremely important, the inducing signal itself plays an equally critical role in cell fate commitment; therefore the inductive ability of embryonic tissue has also received enormous attention in developmental biology. Transplantation has served as an effective technique to address the problem. By grafting donor tissues at various time points and into various locations near the host tissue that is known to be competent, the host response could reveal the potency as well as the spatial and temporal limit of the inducing signal.

Perhaps the most famous transplantation experiment in the history of developmental biology is the organizer experiment. Following the transplantation of the dorsal lip region (organizer) at early gastrula stage to the ventral region of another amphibian embryo, the donor organizer induces the formation of a secondary embryo (Fig 3). This was the first time that a specific embryonic tissue was shown to possess the ability to induce neural fate and organize a new, duplicated axis on the ventral side of the embryo. In amphibians, the organizer itself is shown to be induced by the Nieuwkoop center at the dorsal vegetal pole, as transplanted dorsal vegetal blastomeres are capable of axis induction. This ability is likely the result of dorsal-inducing material in the cytoplasm near the vegetal cortex.

A similar experiment was performed on avian embryos by Waddington several years later, revealing that the anterior region of the primitive streak, known as the Hensen's node, has the same neural inducing ability as the amphibian organizer.

The shield region in fish was discovered to be analogous to the organizer. While the mouse node was long thought to possess the organizer ability, it wasn't experimentally confirmed until 1994. Organizer tissue largely gives rise to mesoendodermal tissues while the resulting neuroectoderm is mainly of host origin. The inductive ability of the organizer is largely dependent on age, as the frequency of neural induction declines with advancing donor age.

The age of the organizer is an important determinant of the type of neural tissue induced. In chick embryos, young nodes (HH stages 2–4) are capable of inducing both anterior and posterior neural structures, while older nodes (HH stages 5–6) tend to have less overall inducing ability and are able to give rise to posterior nervous system. This is consistent with the Nieuwkoop activation-transformation hypothesis, where a wave of activation initially induces the presumptive neuroectoderm to an anterior neural fate, and a transforming factor then posteriorizes the already neuralized tissue. Surprisingly,

one study found that the chordoneural hinge and the tip of the tail retain Spemann's tail organizer activity even during tadpole stages of development.

Molecular analysis revealed that the "inducing capacity" of the organizer was actually attributable to the inhibition of the bone morphogenetic protein (BMP) pathway by BMP-inhibiting factors within the organizer such as chordin, noggin, and follistatin. The dependency of organizer's dorsalizing ability on these factors was demonstrated using transplantation combined with molecular techniques. When chordin expression in donor embryo is knocked down, the organizer failed to induce neural tissue in host embryo. The importance of chordin was further demonstrated when embryos transplanted with Spemann organizer tissue in the ventral side produced a second gradient of Chordin. Another important contributor to neural induction is the fibroblast growth factor (FGF) signalling pathway, since a defective FGF pathway was shown to severely interfere with induction.

The various transplantation experiments led to widespread acceptance of the ability of organizers to induce neural tissue. However, other tissues have been proposed to possess the ability of neural induction, though these views are more controversial. One such idea is the homeogenetic induction of neural tissue, which refers to the induction of neural tissue by existing neuroectoderm. This phenomena was first reported by Waddington in avians, when a neural plate graft was able to induce host neural plate formation. This ability was further demonstrated in *Xenopus* when the presumptive neural plate of late gastrulae induced further neural structures in competent early gastrula ectoderm.

In addition to organizer and homeogenetic induction, the endoderm was also proposed to have neural inductive ability. Waddington observed that rotating the endoderm typically impeded the lengthening of the primitive streak, or triggered the development of a new primitive streak in chicks. However, it wasn't completely clear if this was caused by the neural inductive ability of the endoderm or simply the ability for endoderm to induce cell movement that leads to the development of the primitive streak. Both camps have found evidence that support their own argument. After rotation of the hypoblast by 90° in chicks at stage XIII, the direction of the primitive streak was according to the orientation of the hypoblast, whereas at HH stage 3, it gradually shifted towards that of the epiblast, thus identifying a window of hypoblast inductive ability.

The induction of lens, neural crest, notochord, placode, floor plate, primary mouth, Rathke's pouch, and even the three germ layers have all been addressed using transplantation techniques. Combined with other techniques, transplantation has significantly increased our understanding of the inductive ability of embryonic tissues during early development.

Case study: Waddington experiment

Waddington wanted to study induction in greater detail. The limit to these studies had been the amount of time an embryo could be successfully cultivated ex vivo. Waddington applied *in vitro* cell culturing techniques to this experiment, as opposed to the chorio-allantoic technique used in many earlier studies. Culturing *in vitro* consisted of placing the embryo on a clot of adult chicken blood plasma and chick embryo extract in a watch glass. Experiments reported in this paper were divided into three main sections:

- The development of the embryos *in vitro*,
- Induction by the endoderm, and
- Induction by the primitive streak.

The development of the embryos in vitro was slower and less complete than normal development. Development also failed to continue beyond two days after cultivation. Waddington made no effort to improve the time of development or determine the degree of slowdown in this study because these

limitations did not impact the results of this experiment. Another complication was that the endoderm also had a tendency to form cysts during in vitro cultivation. However, Waddington was able to cultivate the embryos long enough to observe primitive streak formation, gastrulation, and the induction of neural tissue. This work provided the foundation for the experiments in this study.

Waddington first focused on the primitive streak. Waddington's first test was to cut the streak in various places and observe the effect of these manipulations on gastrulation. He discovered that

- A cut anterior to the primitive pit, or Hensen's node, still allowed the formation of many structures. The further back the cut was applied, the fewer structures developed in the embryo.
- Once Waddington cut posterior to the middle of the primitive streak, no more structures developed.
- The influence of the endoderm on the primitive streak was studied to determine whether the endoderm had any organizing effects during development. First, Waddington removed the endoderm from the rest of the embryo and allowed both to develop separately. Differentiation still occurred in the remaining embryo, but Waddington was not satisfied with this result; some endodermal material may have remained on the embryo, causing induction.

His next experiment was to **rotate the primitive streak relative to the axis of the endoderm**. This produced embryos with bent primitive streaks. As the primitive streak developed, it changed direction to follow the axis of the endoderm, demonstrating the formative influence on the primitive streak by the endoderm.

- In order to study the effects of the mesoderm on the developing system, Waddington placed an inverted embryo in the primitive streak stage on another embryo of a similar stage so that the primitive streaks faced each other. This setup was adapted to different angles between the axes of each embryo.
- In many cases, the ectoderm of the upper embryo formed two neural plates instead of the normal one. These plates were formed in the same directions as both primitive streaks.

Waddington's conclusion from this experiment was that the ectoderm is competent to form neural plates in any orientation, demonstrating induction of neural tissue by the primitive streak.

Waddington's final experiment was a **grafting experiment** (Fig 4).

- In this experiment, a primitive streak from one embryo was grafted to another embryo. Grafts were made from different thirds of the primitive streak. The anterior third of the streak, including the primitive node, was shown to induce neural tissue in the host ectoderm.
- The posterior third of the streak never induced neural tissue, implying that the posterior portions of the primitive streak are not capable of induction.
- In these experiments, Waddington identified the anterior portions of the primitive streak to be the organizing tissue.

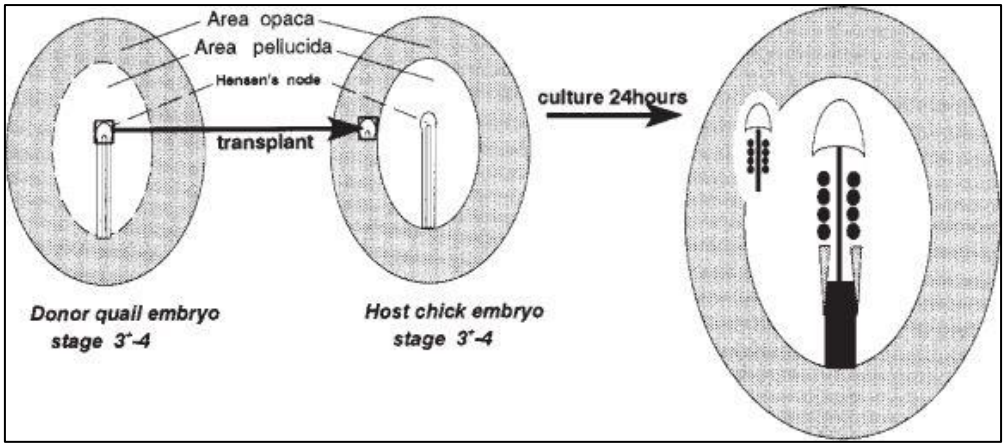


Fig 4: Grafting Henson's node

He first improved the cultivation of birds in vitro and then performed experiments on the primitive streak. He sliced the streak at various positions to show that by disrupting gastrulation before the anterior portions of the streak, induction of many structures failed. He also showed that the endoderm had a formative influence on the primitive streak, since the primitive streak followed the axis of the endoderm. He demonstrated that the primitive streaks of two blastoderms arranged adjacent to each other at varying angles could induce similarly oriented neural tissue. He showed that grafts from one embryo into a pocket between the endoderm and ectoderm could induce a second axis in the host.

Waddington used these experiments to show that the higher vertebrates did have tissues equivalent to an organizer. This tissue was centred on Hensen's node, demonstrating that higher vertebrates had similar developmental mechanisms to the previously studied amphibians.

Embryonic plasticity

The ability of a cell, tissue or organ to react to an external input or injury by altering its state or even its fate is known as plasticity. It reveals the potential of the cell and its regulative capacity. Regeneration is often associated with plasticity. External input including physical, chemical, genetic, and extreme temperature. Transplantation has become one of the most common ways to understand 'embryonic plasticity' following physical perturbation, as it allows the study of plasticity on both the tissue level and the individual cellular level. Plasticity is quite obviously related to the state of determination given that determined tissues will fail to show regulation.

Though most types of embryonic tissue possess some degree of plasticity, transplantation studies have been primarily focused on the plasticity of anterior-posterior (AP) neural axis as well as that of neuronal innervation. Earlier embryological studies on AP neural axis plasticity examined the plasticity of the entire neuroectoderm. The main technique employed was the 180˚ rotation transplant of the neuroectoderm along the AP axis of the embryo. By assaying the recovery of the transplant embryo, as well as the regional identity of transplant tissue at a later stage, the degree of AP neural plasticity can be revealed. If the anterior-posterior identity' of cells in the transplant tissue follows the AP pattern of the host, it suggests that the AP characteristics in the donor neuroectoderm was not yet determined; on the other hand, if cells in the rotated tissue adopt anterior-posterior identity according to their original AP orientation, it is likely that the donor embryo has lost its AP neural axis plasticity. This period during which the neuroectoderm is competent to respond to signals from host tissue and adopt new AP regional identity is referred to as the window of plasticity. When Spemann performed AP neural axis rotation for the first time, he found that neural plate stage embryos have lost their AP neural plasticity. However, this result should be treated with caution because the inductive ability of

mesoderm wasn't fully understood at the time, and Spemann rotated the neuroectoderm along with the underlying mesoderm.

The transplantation of *Xenopus* prospective spinal neuroectoderm tissue to presumptive eye and prosencephalic regions at neural plate stage found a mixture of anterior and posterior features at the transplant regions, while the transplantation of anterior neural plate to more posterior positions showed full caudalization at stage 11/12 and no caudalization at stage 16, thus corroborating this conclusion.

Fate mapping of early embryonic tissue

Tissue transplantation has also served as an invaluable tool for studying the movements and fates of tissues throughout development. This form of fate mapping often involves isotopic and homochronic transplantation of tissue from donor to host, and it is advantageous because donor cells and their derivatives can be easily distinguished from host cells, facilitating an accurate mapping of transplanted tissue fate. Among the most well studied tissue systems in developmental biology is the embryonic neuroectoderm. Transplantation studies have been utilized to elucidate both general and detailed information regarding the developmental fate of various neural structures. The neural crest, for instance, has been a well-studied structure in various organisms and in a variety of detail. Rosenquist utilized transplantation methods in labelled chick embryos to confirm the origin of neural crest cells as the neural/non-neural border in the anterior epiblast. Later work in *Xenopus* embryos has mapped neural crest cells in more detail, using interspecies transplants of *Xenopus borealis* donor cells within host *Xenopus laevis* embryos to study different segments of the neural crest and their derivatives.

Other transplantation studies on the anterior pituitary and telencephalon have mapped the origin and derivatives of these structures in greater detail. The ventral neural ridge, for instance, was elucidated as the origin of the anterior pituitary through isotopic-labeled donor transplants in *Xenopus laevis*. Similar transplants of the neural ridge were done using quail-chick chimera transplants, revealing that the posterior pituitary originates in a region of the neural folds separate from the anterior pituitary and that these two regions are separated by the presumptive hypothalamus. Quail-chick chimera transplants were also utilized to perform transplants of the telencephalon to locate the telencephalic subpallium as the origin of inhibitory neurons invading the pallium.

Tissue transplantation during early embryonic development has also shed light on the origins of blood cells in haematopoiesis. Yolk sac transplants between chick and quail embryos has indicated that hematopoietic cells are of yolk sac origin, including a group of macrophage-like cells that partake in an early embryo phagocytic cell system of blood cell lineage, and endothelial cells are of intraembryonic origin.

Experiment

A group of cells is called specified if, when isolated and cultured in the neutral environment of a simple culture medium away from the embryo, they develop more or less according to their normal fate.

For example cells at the animal pole of the amphibian blastula are specified to form ectoderm and will form epidermis when isolated. Cells that are specified in this technical sense need not yet be determined for influences from other cells can change their normal fate; if tissue from the animal pole is put in contact with cells from the vegetal pole, the animal pole tissue will form mesoderm instead of epidermis.

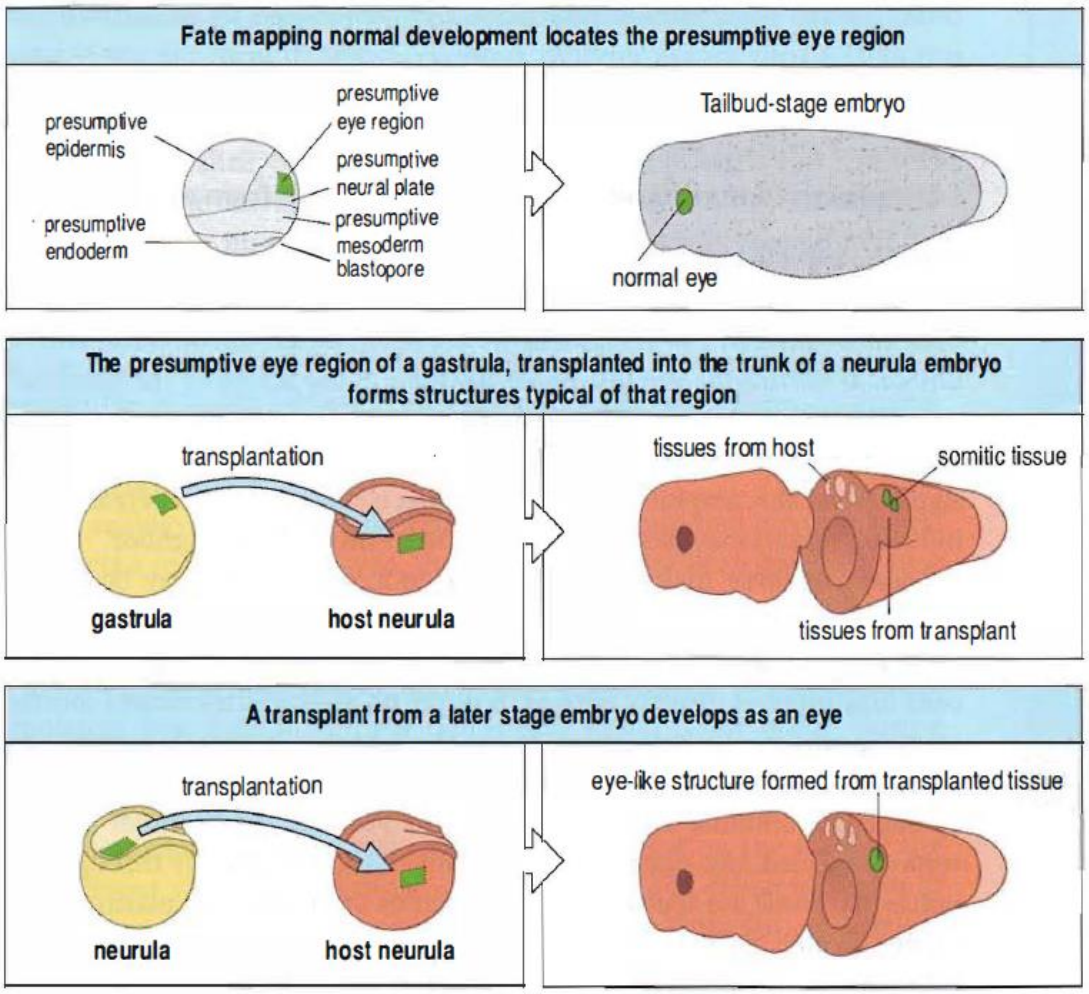


Fig 5: Determination of the eye region with time in amphibian development. If the region of the gastrula that will normally give rise to an eye is grafted into the trunk region of a neurula (middle panel), the graft forms structures typical of its new location, such as notochord and somites. If, however, the eye region from a neurula is grafted into the same site (bottom panel). It develops as an eye-like structure, since at this later stage it has become determined.

At a later stage of development, however, the cells in the animal region have become determined as ectoderm and their fate cannot then be altered. Tests for specification rely on culturing the tissue in a 'neutral' environment lacking any inducing signals, and this is often difficult to achieve. The state of determination of cells at any particular stage can be demonstrated by transplantation experiments. At the blastula stage of the amphibian embryo, one can graft the ectodermal cells that give rise to the eye into the side of the body and show that the cells develop according to their new position; that is, into mesodermal cells like those of the notochord and somites (Fig 5). At this early stage, their potential for development is much greater than their normal fate. However, if the same operation is done at a later stage, then the future eye region will form structures typical of an eye. At the earlier stage the cells were not yet determined as eye cells, whereas later they had become so.

Analysis of gene function

It is clear from the studies described in previous sections that the use of molecular markers allowed precise assessment of the state of determination and competence of tissues as well as their inducing capacity. However this technique has also served as an invaluable and essential tool in delineating the role of genes in particular developmental processes. A specific method of transplants, termed mosaic

analysis, involves transplanting a group of cells (typically 10–50 cells) from embryos with a mutant form of a gene to a wild-type embryo or vice versa and analysing the behaviour of the transplanted cells in the new environment. This can reveal whether a gene function is cell autonomous or cell non-autonomous, and elucidate in conjunction with additional markers the effect of the mutant gene on surrounding tissues. In this way, the contribution of specific genes within a developmental pathway can be observed separately, and then analysed collectively. Mosaic analysis has most commonly been employed in genetically tractable models such as the zebrafish, as its robustness, accessibility, and relative transparency during embryogenesis facilitate the observation of interactions among individual cells. This method has been employed in the study of a variety of different developmental processes that include mesoendodermal specification, haematopoiesis, hindbrain development, and cell movements during gastrulation.

Mosaic analysis methods have been widely employed in the study of specific genes involved in hematopoiesis. Zebrafish swirl mutants, embryos that contain a mutated form of bmp2b, display expanded dorsal structures including notochord and somites, while more ventral structures including blood and nephros are absent. Cell transplants between wild-type zebrafish embryos and swirl mutants have implicated bmp2b as a non-autonomous contributor to blood cell specification, indicating that bmp2b acts within the BMP pathway as a ligand rather than a receptor or downstream signal transducer. The smadS (sbn) gene acts downstream of bmp2b, and mutants exhibit similar dorsalized phenotypes to the swirl mutants. Mosaic analysis of these mutants, termed somitabun mutants, has revealed that the action of sbn in the specification of ventral derivatives is non-autonomous. This suggests that the bmp2/4 function during dorsoventral patterning is two-fold, with an initial sbn-independent phase and a later independent phase, as sbn is required cell-autonomously for the downstream autoregulation of bmp2b in dorsoventral patterning before its non-autonomous role in the specification of blood cells and other ventral cell types.

Heterotopic transplants at the marginal zone between mutant and wild-type zebrafish for the cloche gene have revealed that the gene acts autonomously and non-autonomously during hematopoiesis. Transplants of wild-type cells to mutant hosts resulted in both wild-type and mutant cells expressing gatal, a genetic marker in red blood cells; however, wild-type cells were still far more likely to contribute to blood cells than mutant cells. This phenomenon suggests that cloche is required non-autonomously for expression of gatal during blood cell differentiation, but is required cell-autonomously in blood cells for their subsequent proliferation and survival following gatal induction.

Cell transplants between wild-type and mutant zebrafish embryos of notochord-inducing genes floating head and no tail have indicated that these genes are required autonomously for notochord formation via the maintenance of notochord-inducing axial mesoderm and the differentiation of notochord precursors, respectively. The generation of mosaic zebrafish embryos for both notochord (ntl,flh, and doc) and prechordal mesoderm (cycl and oep)-inducing genes have additionally indicated that, contrary to previous beliefs, floor plate cells are induced largely by the prechordal and axial mesoderm rather than the notochord, which require non-autonomous expression of neekless for their own formation.

In addition to the major areas studied by mosaic analysis, cell transplants have also been employed to explore craniofacial development, eye development, cellular movements, and other phenomena. Mosaic analysis has implicated the zebrafish lockjaw gene non-autonomously in the formation of mesodermally-derived muscles, skeletogenesis, and melanophore development as well as autonomously in the migration and contribution of neural crest cells to the pharyngeal arches [168–170]. Both zebrafish casanova and chinless genes have also been implicated in pharyngeal arch development, although cell transplants have indicated that cas requirement for pharyngeal cartilage

formation is environmental, while chinless exhibits both autonomous and non-autonomous roles in cartilage and muscle formation.

Future directions

- It is clear from the experiments described in the preceding sections that embryonic tissue transplantation has had a major impact on the field of developmental biology beginning with the birth of experimental embryology and carrying through to discoveries that relied on the use of precise gene expression assays for identifying cell identity and unambiguous host and donor markers.
- Tissue transplantation has led to a clear delineation of the state of determination or commitment of each tissue and presumptive organ in a wide variety of organisms, and has enabled the identification of genes that confer this state of near irreversible commitment. Likewise this approach has defined the competence of tissues to respond to inducing signals, as well as the ability of specific tissues and cells to provide inducing signals.
- Transplantation has allowed precise fate-mapping of groups of cells and even individual cells with impressive resolution throughout development and has been instrumental in investigating the molecular basis of plasticity, an emerging field relevant for regenerative medicine.
- Finally, the ability to transplant tissues and even single cells carrying a specific mutation into a wild type embryo or wild type cells into an embryo with a mutant background has provided key information on the functions of hundreds of key developmental genes, delineating whether a particular gene acts cell autonomously or not.
- However while transplantation experiments have been essential for past insights in developmental biology, with newer technologies and emerging areas of interest, the use of tissue transplantation has the potential to keep providing new insights regarding embryonic development.
- Applying the power of RNA-Seq, particularly single cell RNA-Seq, to transplantation studies, could identify global responses to transplantation and identify key transcriptional networks that re-pattern both the donor and neighbouring host cells. Excitingly, recent advances in RNA-Seq technology like tomo-seq make it possible to acquire transcriptional information in 3D, thus integrating gene expression profiles with embryonic patterns.

Conclusion

Transplantation during early embryonic stages is a classical technique in developmental biology that has significantly contributed to our understanding of a plethora of issues. Through a variety of transplantation schemes, scientists have made significant breakthrough in understanding the timeline of specification and determination, inductive ability and competence, as well as the eventual fate of diverse embryonic tissue during early embryonic development. Its use has spanned most common vertebrate model organisms, organ and tissue types, and developmental stages. Modern molecular techniques have allowed more accurate and mechanistic investigation of embryonic development using transplantation. In addition to novel uses such as single-gene analysis, these new techniques will allow for the reinvestigation and expansion of earlier transplant studies, thus paving the way for discoveries of greater detail and accuracy in the field. With the rapid advancement of global genomic and transcriptomic analysis methods such as RNA-Seq and differential gene expression analysis, it is now possible to comprehensively examine the molecular nature of embryonic responses to tissue transplantation. Such analysis should become the direction of future transplantation studies on early embryos, as it will further contribute to the transition of early embryonic transplantation studies from descriptive to the quest of underlying molecular mechanisms. Moreover, the integration of such techniques with regenerative medicine would have significant impacts on basic and clinical science.

Probable questions:

1. Discuss the determination and competence of embryonic tissues.
2. Describe the Spemann-Mangold organizer experiment.
3. Describe the inductive ability of embryonic tissue.
4. Describe Waddington experiment
5. What is primitive streak?
6. Discuss briefly embryonic plasticity.

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Unit III

Growth: Definition, Relative growth of parts, growth gradients

Objective: In this unit you will know about Growth; its definition, relative growth of parts and growth gradients

Introduction

Growth, the increases in cell size and number that take place during the life history of an organism. Grey 1931 speaks of growth as "essentially concern with the formation of new living material". Medawar 1941 states that "what result from the biological growth is itself typically capable of growing". Weiss 1949 defined growth as "the increase in that part of the molecular population of an organic system which is synthesized within that system" and it means "the multiplication of that part of the molecular population which is capable of further continue reproduction". Thus in order to formulate a precise concept of growth it should be confined to the increase the amount of this system which is capable of growing. The main problem which is required the study is-

1. at what rate does this increased takes place.

2. How does the rate changes at time passes.

Fig 1: Growth stage; various growth stages of the Emperor Gum moth caterpillar (*Opodiphthera eucalypti*).

The process of growth

Growth is seldom random. Rather, it occurs according to a plan that eventually determines the size and shape of the individual. Growth may be restricted to special regions of the organism, such as the layers of cells that divide and increase in size near the tip of the plant shoot. Or the cells engaged in growth may be widely distributed throughout the body of the organism, as in the human embryo. In the latter case, the rates of cell division and of the increase in cell size differ in different parts. That the pattern of growth is predetermined and regular in plants and animals can be seen in the forms of adults. In some organisms, however, notably the slime molds, no regular pattern of growth occurs, and a formless cytoplasmic mass is the result.

The rate of growth of various components of an organism may have important consequences in its ability to adapt to the environment and hence may play a role in evolution. For instance, an increase in the rate of growth of fleshy parts of the fish fin would provide an opportunity for the fish to adapt more easily to terrestrial locomotory life than could a fish without this modified fin. Without disproportionate growth of the fin—ultimately resulting from random changes in the genetic material (mutations)—the evolution of limbs through natural selection might have been impossible.

Types of growth

In cells

The increase in size and changes in shape of a developing organism depend on the increase in the number and size of cells that make up the individual. Increase in cell number occurs by a precise cellular reproductive mechanism called mitosis. During mitosis the chromosomes bearing the genetic material are reproduced in the nucleus, and then the doubled chromosomes are precisely distributed to the two daughter cells, one of each chromosomal type going to each daughter cell. Each end of the dividing cell receives a complete set of chromosomes before the ends separate. In animal cells this is a pinching off (cytokinesis) of the cell membrane; in plant cells a new cellulose wall forms between the new cells. During the period of cell life preceding the actual distribution of chromosomes, the mother cell often grows to twice its original size. Hence, a cycle consisting of cell growth and cell division is established.

Cell growth—an increase in cytoplasmic mass, chromosome number, and cell surface—is followed by cell division, in which the cytoplasmic mass and chromosomes are distributed to the daughter cells. An increase in cytoplasmic mass does not always occur during cell-division cycles, however.

During the early development of an embryo, for example, the original egg cell, usually a very large cell, undergoes repeated series of cell divisions without any intervening growth periods; as a result, the original egg cell divides into thousands of small cells. Only after the embryo can obtain food from its environment does the usual pattern of growth and mitosis occur.

In plants

The fact that most plant cells undergo extensive size increase unaccompanied by cell division is an important distinction between growth in plants and in animals. Daughter cells arising from cell division behind the tip of the plant root or shoot may undergo great increases in volume. This is accomplished through uptake of water by the cells; the water is stored in a central cavity called a vacuole. The intake of water produces a pressure that, in combination with other factors, pushes on the cellulose walls of the plant cells, thereby increasing the length, girth, and stiffness (turgor) of the cells and plant. In plants, much of the size increase occurs after cell division and results primarily from an increase in water content of the cells without much increase in dry weight.

The very young developing plant embryo has many cells distributed throughout its mass that undergo the cycle of growth and cell division. As soon as the positions of the root tip, shoot tip, and embryonic leaves become established, however, the potential for cell division becomes restricted to cells in certain regions called meristems. One meristematic centre lies just below the surface of the growing root; all increases in the number of cells of the primary root occur at this point. Some of the daughter cells remain at the elongating tip and continue to divide. Other daughter cells, which are left behind in the root, undergo the increase in length that enables the new root to push deeper into the soil. The same general plan is evident in the growing shoot of higher plants, in which a restricted meristematic region at the tip is responsible for the formation of the cells of the leaves and stem; cell elongation

occurs behind this meristematic centre. The young seedling secondarily develops cells associated with the vascular strands of phloem and xylem—tissues that carry water to the leaves from the soil and sugar from the leaves to the rest of the plant. These cells can divide again, providing new cell material for development of a woody covering and for more elaborate vascular strands. Hence, the growth of higher plants—*i.e.*, those aspects involving both the pattern of stems, leaves, and roots and the increase in bulk—results primarily from cell division at the meristem followed by a secondary increase in size because of water uptake. These activities occur throughout the period of plant growth.

In animals

The growth of animals is more restricted in time than is that of plants, but cell division is more generally distributed throughout the body of the organism. Although the rate of cell division differs in different regions, the capacity for cell division is widely distributed in the developing embryo. Increase in size is rapid during the embryonic period, continues at a reduced rate in juveniles, and thereafter is absent. Cell division and size increase continue, however, even after increase in total body size no longer occurs. Because these events are balanced by cell death, post-juvenile increase in cell number is primarily a replacement phenomenon. Height increase in mammals is limited by cessation of cell division and bone deposition in the long bones. The long juvenile period of growth in humans is unusual, higher animals attaining mature size soon after the end of embryonic development. Some organ systems undergo little cell division and growth after birth; for instance, all of the germ cells (precursors of egg cells) of the female are formed by the time of birth. Similarly, all of the nerve cells of the brain are formed by the end of the embryonic period. Further increase in the size of the nervous system occurs by outgrowth of nerve fibres and deposition of a fatty insulation material along them. Although the greatest increase in size of nerve cells occurs, as in plant cells, after the cessation of cell division, the nerve fibre outgrowth in animals represents a true increase in the amount of cytoplasm and cell surface and not just an uptake of water.

Some organs retain the potential for growth and cell division throughout the life span of the animal. The liver, for example, continues to form new cells to replace senescent and dying ones. Although cell division and growth occur throughout the liver, other organs have a special population of cells, called stem cells that retain the capacity for cell division. The cells that produce the circulating red cells of mammalian blood are found only in the marrow of the long bones. They form a permanent population of dividing cells, replacing the red cells that continuously die and disappear from the circulation.

The rates of both growth and cell division can vary widely in different body parts. This differential increase in size is a prime factor in defining the shape of an organism.

Normal and abnormal growth

- **Tumours**

When growth is not properly regulated, anomalies and tumours may result. If the increase in the number of liver cells is abnormal, for example, tumours of the liver, or hepatomas, may result. In fact, one feature of malignant tumours, or cancers, is the absence of the usual growth patterns and rates. The cells of malignant tumours, in addition to having abnormal growth rates, have altered adhesive properties, which enable them to detach easily from the tumour; in this way the cells may spread to other parts of the body (metastasize) and grow in unusual locations. It is the growth of tumours in places other than the organ of origin that usually causes the death of an organism. Tumours may vary widely in their growth rates. They may grow very rapidly or so slowly that the rate approaches that of normal cell division in adult tissues. Tumours are not only characterized by an increase in the rate of

cell division but also by abnormal patterns of growth. The new cells formed in the tumour are not organized and incorporated into the structure of the organ and may form large nodules. These abnormal growths may present no medical problems (*e.g.*, moles) or may cause disastrous effects, as is the case of the pressure on the brain caused by a tumorous mass of the meningeal covering of the brain.

- **Regeneration**

Not all abnormal growths are tumours. If a tree is partially burned, cells below the bark produce a new covering for the exposed vascular strands. Growth may not be normal, and an obvious scar or growth of the new bark is apparent. Similarly, if the skin of a mammal is severely injured, the repair, although abnormal and imperfect, causes the organism no physiological difficulty. Many organisms possess the ability to regrow, or regenerate, with varying degrees of perfection, parts of the body that are lost or injured. Salamanders possess remarkable powers of regeneration, being able to form new eyes or a new limb if the original is lost. Lizards can regenerate a new tail; even humans can regenerate parts of the liver. The reasons for the differences in regenerative powers in different animals remain a fascinating mystery of great practical importance. When regeneration does occur, some specialized cells usually lose their specialized characteristics and enter a period of an increased rate of cell division; subsequently, the new cells respecialize into the tissues of the original body part. Plants whose tops are lost as in pruning can also sometimes form new meristematic centres from dormant tissues and produce new shoots.

Compensatory growth

Many organs of animals occur in pairs, and if one is lost the remaining member increases in size, as if responding to the demands of increased use. If one of the two kidneys of a human is removed, for example, the other increases in size. This is called a compensatory reaction and may occur either by some increase in cell size (hypertrophy), by an increase in the rate of cell division (hyperplasia), or both. Although an increase in cell number is primarily responsible for the compensatory reaction of the kidney, the number of individual filtration units (glomeruli) does not increase. Hence, cell division increases the size of glomeruli but not the total number. Some of the most striking examples of increases in cell size in animals take place during stimulation of endocrine organs, which secrete regulatory substances called hormones; when the thyroid gland is stimulated, for example, the individual cells of the gland may increase dramatically in size.

Types of development

Biological development, the progressive changes in size, shape, and function during the life of an organism by which its genetic potentials (genotype) are translated into functioning mature systems (phenotype). Most modern philosophical outlooks would consider that development of some kind or other characterizes all things, in both the physical and biological worlds. Such points of view go back to the very earliest days of philosophy. In the entire realm of organisms, many different modes of development are found, the most important categories of which can be discussed as pairs of contrasting types.

- **Single-phase and multiphase development**

The most familiar organisms, including man, undergo a single-phase development; the organs that appear at early stages persist throughout the whole of life. There are many kinds of animals that develop one or more larval stages adapted to a life different from that of the adult. Perhaps the best known of these is the common frog.

The egg first develops into a tadpole, which is provided with a large muscular tail by which it swims. The tadpole eventually undergoes a change of form, or metamorphosis. This involves the regression and resorption of the tail and the growth of the limbs. During this time the rest of the body of the tadpole undergoes less profound changes; the organs persist but undergo relatively far-reaching progressive changes.

Plants in general appear to exhibit a type of development related in a general way to the multiphase development just discussed in animals, although rather different from it in essence. This is called the "alternation of generations." The majority of higher plants possess two sets of similar chromosomes in each of their cells, that is to say they are diploid (2*n*), as are most higher animals. But in sexual reproduction, diploid cells undergo a reduction division so as to form precursors of the sex cells, which are haploid—*i.e.,* they contain only one set of chromosomes. In animals these cells develop directly into the sex cells—egg and sperm—which unite in fertilization. In plants the haploid cells undergo some developmental processes before the functioning sex cells are produced. The products of this development are spoken of as the "haploid generation." In most higher plants the haploid development is quite reduced, so that the haploid individuals contain only a few nuclei—those associated with the pollen tube on the male side and a few associated with the egg on the female side. In some lower plants, however, such as mosses and ferns, the haploid development may be much more extensive and give rise to quite sizable separate plants. In such cases a species contains two kinds of individuals, produced by different types of developmental processes controlled, however, by the same genotype. This may be compared with the multiphasing development of larval forms in animals. The situation in plants, however, is characterized by the two forms of the organism having different chromosomal constitutions—haploid and diploid—whereas the larval forms and the adult of an animal species have the same chromosomal constitution.

- **Structural and functional development**

Function is the capacity of the biological system to carry out operations. At the level of the organism, these operations include walking, swimming, eating, digesting, etc.; at the cell level, typical functions are respiring, contracting, conducting nervous impulses, secreting hormones, etc.; and at the molecular level, all functions depend on the production of enzymes, coded by particular genes. Structure encompasses all parts of the organism capable of carrying out functions localized within the body of the organism and arranged in some particular spatial pattern. Contractile cells, for example, are grouped together to form muscle, and other cells are grouped together to form elements of the skeleton; both the muscles and the skeletal elements have definite spatial relations to each other.

It is, however, often convenient to focus discussion on one or other of these two aspects of development; for instance, a study may be made into the developmental processes that bring about the production of haemoglobin or insulin by a certain kind of cell, without at the moment being concerned with structural problems. Or again, the focus may be on the results of a certain process by which a mass of cells develops into a typical hand with five digits. In such an inquiry the structural aspects are paramount.

Factors That Regulate Growth

A. Environmental factors

The environment in which an organism lives plays an important role in modifying the rate and extent of growth. Environmental factors may be either physical (*e.g.,* temperature, radiant energy, and atmospheric pressure) or chemical.

- **Temperature**

Organisms and the cells of which they are composed are extremely sensitive to temperature changes; as the temperature decreases, the biochemical reactions necessary for life occur more slowly. A lowering of the temperature by 10° C (18° F) slows metabolism at least twofold and often more.

Temperature affects both warm- and cold-blooded animals. Many warm-blooded (e.g., bears) and cold-blooded (e.g., frogs) vertebrates cease growing during the cold winter and simply enter an inactive or dormant state, which is characterized by a very low rate of metabolism. In animals that do not become dormant, increased demands for food consumption occur during cold periods to provide energy to maintain body temperature; this utilization of food energy may limit the energy available for size increase if food is in short supply.

- **Pressure**

Because atmospheric pressure is relatively constant except in the mountains, it probably is of little importance in growth regulation. Increases in pressure in the ocean's depths may be significant, however, since it is known that increases in hydrostatic pressure interfere with cell division. Tissues of deep-sea fishes must have become adapted to such pressure effects, which have been little studied thus far. Movements of the terrestrial atmosphere—winds—may affect growth patterns in trees and shrubs, as is evident in the exotic shapes of certain conifers that grow along coastlines exposed to strong prevailing winds.

- **Light**

Among all the physical factors, light plays the best understood and most dramatic role. Many of the effects of light on plant growth are obvious and direct. Light energy is the driving force for photosynthesis. Insufficient light causes death or retardation of growth in green plants. But light also has indirect effects of great importance. Green plants possess small amounts of a pigment called phytochrome that can exist in two forms. One form absorbs red light (660 millimicrons, or mμ; 1 mμ = 3.937×10^{-8} inch). When plants containing this pigment absorb red light, the pigment is converted to another form, which absorbs far-red light (730 mμ); the latter form can be converted back again to the original red absorbing form. These conversions have dramatic consequences; for example, red light inhibits stem elongation and lateral root formation but stimulates leaf expansion, chloroplast development, red flower coloration, and spore germination. Cycles of red and far-red light also can affect flower formation.

B. Chemical factors

Chemical factors of importance in the environment include the gases in the atmosphere and the water, mineral, and nutritional content of food. Plants require carbon dioxide, water, and sunlight for photosynthesis; drought slows plant growth and may even kill the plant. The effects of atmospheric contaminants—e.g., oxides of nitrogen, hydrocarbons, and carbon monoxide—are known to have deleterious effects on the growth and reproduction of both plants and animals.

Plants and animals require minerals and small amounts of elements such as zinc, magnesium, and boron. Nitrogen and phosphorus are provided to plants as nitrates and phosphates in the soil. Inadequate quantities of any nutritional factor in the soil result in poor plant growth and poor crop yields. Animals require oxygen, water, and elements from the environment. Because they are unable to synthesize sugars from carbon dioxide, animals must acquire these nutrients through the diet, either directly, by the consumption of plants, or indirectly, by the consumption of other animals that in turn have utilized plants as food. If the quality or quantity of this food is poor, either growth is retarded or death occurs

Vitamins, a class of compounds with a variety of chemical structures, are needed by animals in small amounts. Animals cannot synthesize all vitamins they require; those that cannot be synthesized must therefore be acquired in the diet, either from plants or from other animals that can synthesize the vitamin. Because certain vitamins are necessary in certain important metabolic reactions, vitamin deficiency during growth may have a variety of effects—stunting, malformation, disease, or death.

C. Internal factors

The organism is dependent on the environment for the raw materials for growth, but growth is also regulated internally. Because the size and form of plants and animals are under genetic control, events such as the rate and site of cell division and the extent of cell enlargement can be affected by mutations. It is not yet known, however, precisely how these factors, which are the ultimate determinants of growth, are controlled in individual cells.

- **Hormone**

One very important class of intrinsic growth regulators is that of the hormones. Cytokinins and gibberellins in plants influence the rate of cell division in the meristems. Some dwarf plants can be stimulated to grow to normal size simply by applying gibberellin. Auxin somehow changes the characteristics of the rigid cell wall of the plant cell so that it becomes more flexible. Hormones also play a decisive role in animal growth. One hormone from the pituitary gland at the base of the brain is called growth hormone because of its extensive and widespread effects on growth. A deficiency of growth hormone in pre-adolescents results in dwarfism, and oversupply of the hormone (often caused by a tumour) results in gigantism. The sex hormones secreted from the pituitary gland interact in a complex way to regulate the growth of the gonads. The gonads in turn produce estrogen and progesterone in females and testosterone in males; these hormones control the development of human secondary sexual characteristics.

- **Protein factor**

In animals, growth stops entirely, except for replacement, after the juvenile period. The limits for both total body size and organ size are probably established by genetic mechanisms. The factors involved in limiting the growth of an organism are not yet definitely known, but evidence indicates that the liver releases into the bloodstream protein molecules that can limit growth of the organ. Thus, one theoretical view is that an organ may produce substances that serve to limit its own growth, thereby establishing a feedback mechanism. A protein called nerve-growth factor is important for the growth of some parts of the mammalian nervous system. If too much of the nerve-growth factor is present, growth of sympathetic nerve fibres is extensive and aberrant. If the nerve-growth factor is eliminated from the body—by injection of an antibody against the factor—the sympathetic nerves wither and disappear.

Other subtle growth regulatory substances specific for various organ systems may eventually be discovered.

a) The Dynamics of Growth

b) Measurement of growth

The mathematical analysis of the *rate of growth* has been a subject of interest for many years. It is based on the rule of cell division: one cell gives rise to two daughter cells. Hence, the theoretical increase in cell number would be a geometric series, in which one cell produces two cells, then four, eight, 16, and so on. In reality, however, the rate of growth is not constant but declines after a period of time, usually because of influences in the environment or because of inherent genetic limitations.

Thus the curve showing the growth of cell populations and of organisms is usually S-shaped, or sigmoid, when growth is plotted against time on a graph. The increase in cell number resulting from cell division accounts for the rising part of the curve; the rate of cell division decreases at the plateau in the curve. The S-shaped growth curve is generally applicable to the growth of organisms. If growth is plotted against time on a logarithmic scale, the early intense growth (called log growth) in the rising phase of the growth curve falls on a straight line.

The rate of growth may be defined by the differential equation

$$v = dW/dt\ (1/W)$$

in which v is the growth rate and W is the weight at any given time, t. The solution of this equation provides a value for relative increase—the increase in weight related to the initial mass of the growing substance. The animal that most closely approaches a constant rate of growth is an insect larva. In most animals the rate of growth declines as the organism becomes larger and older.

Although the S-shaped growth curve describes with fair accuracy the growth of populations of single cells, such as bacteria or cells of higher organisms in tissue culture—the growth in a sterile nutrient environment of cells of tissues from organisms—the growth rates of different parts of whole organisms vary. The relationship of the growth of one part of an organism to that in another part is called *allometry*. Allometry is the mathematical study of the relative growth of parts of an organism. There is often a mathematical relationship between two structures of lengths x and y. such that

$$y = bx^a,$$

where a and b are constants. So when one plots log y against log x, a straight line of slope a is obtained. The slope of the line indicates how much faster y grows than x. An example of this relationship is provided by the relative growth of the abdomen and head in an ant (Fig 2). As the ant grows, the head becomes relatively much larger.

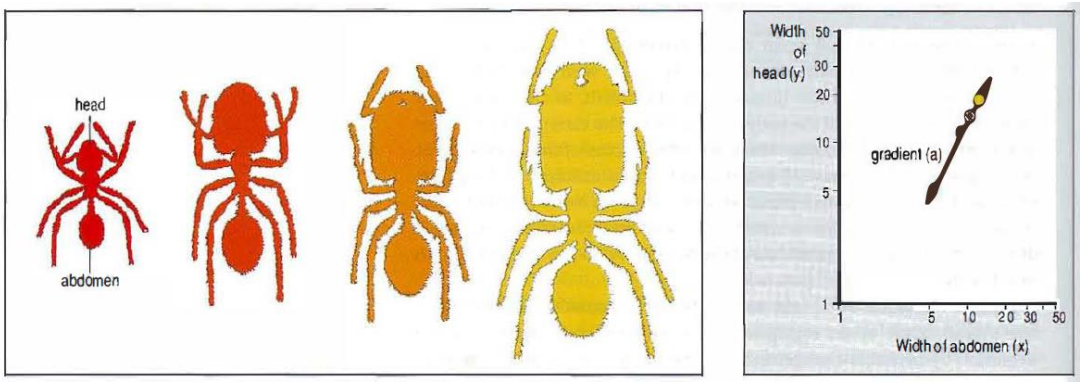

Fig 2: Differential growth of body regions in the ant. As the ant grows, the width of the head (y) increases much faster than that of the abdomen (x) and so the head becomes proportionally larger. The relationship fits the equation $y = bx^a$, where a is the slope of the curve when log y is plotted against log x. and b is a constant.

Although such mathematical tools have allowed a very thorough description of the differential growth of different parts of an organism, they have unfortunately not provided insight into the physical and chemical control of the growth rate.

The study of growth

Even though the chemical, physical, and genetic bases of growth are elusive, much has been learned about the process by growing tissues in a sterile nutrient environment. Even if the source of the tissue is an organ that has completely stopped growing, such as the nervous system of an animal or the phloem of a plant, the cells will begin to grow again in culture, often at a logarithmic rate of increase. It may therefore be concluded that the organism as a whole places constraints upon the ability of individual cells to reproduce and that, when these constraints are removed, the growth potential of the cells is no longer restrained. Even in tissue culture, however, the rate of cell growth eventually slows, hence the sigmoid-shaped growth curve. During the rapid growth phase of cells in tissue culture, they usually lose the ability to carry out the specialized function characteristic of their organ of origin; for example, if cartilage cells divide rapidly, they no longer synthesize cartilaginous matrix.
When the growth of tissue-culture cells begins to slow, one factor responsible is exhaustion of critical components from the medium. But even if the medium is frequently replaced, when the bottom of the culture dish becomes densely packed with a layer of cells, the growth rate drops—a phenomenon called *contact inhibition of growth*. It is believed that cells so close that they are always touching provide a signal that retards the rate of cell division. Apparently identical cells in tissue culture also show great variation in growth rate. Some cells from the skin, for instance, when placed in culture, may divide every eight hours; other similar cells may divide only every 36 hours. The growth of cells in a controlled environment such as tissue culture offers many possibilities for studying the fundamental mechanisms controlling cell growth and, consequently, the growth of organisms and populations.

Relative Growth

Developing systems normally increase in size, at least during part of their development. "Growth" is a general term used to cover this phenomenon. It comprises two main aspects:

(1) Increase in cell numbers by cell division and

(2) Increase in cell size.

These two processes may in some examples occur quite separately from each other; for instance, cells in certain rapidly growing tissues (*e.g.,* the connective tissue or blood-forming systems in vertebrates) may increase greatly in number, while the cells remain approximately the same size. Alternatively, in some organs (*e.g.*, the salivary glands of insects) the cells may increase greatly while remaining the same in number, each cell becoming enlarged, or hypertrophied. In such greatly enlarged cells there is often duplication of the genes, involving an increase in the DNA content of the nucleus, although no cell division takes place, and the nucleus continues as a single body, although with a multiplied, or "polyploid," set of chromosomes.

In very many cases, however, the growth of an organ depends on increases both in cell number and in cell size. The relative importance of these two processes has yet to be properly investigated. One case that has been well studied is the size of the wings of the fruit fly *Drosophila*. The number of cells in the wing can be easily determined, since each bears a single hair that can be seen and counted in simple microscopic preparations. It has been found that there is an accommodation of factors: if there is an unusually large number of cells, these may be somewhat smaller than usual, so that the total size of the wing remains relatively unchanged.

Morphogenesis

Morphogenesis refers to all those processes by which parts of a developing system come to have a definite shape or to occupy particular relative positions in space. It may be regarded as the architecture of development. Morphogenetic processes involve the movement of parts of the developing system from one place to another in space, and therefore involve the action of physical forces, in contrast to processes of differentiation, which require only chemical operations.

There is an enormous variety of different kinds of structures within living organisms. They occur at all levels of size, from an elephant's trunk to organelles within a cell, visible only with the electron microscope. There is still no satisfactory classification of the great range of processes by which these structures are brought into being. The following paragraphs constitute a tentative categorization that seems appropriate for the present state of biological thought on this topic.

Morphogenesis by differential growth

After their initiation, the various organs and regions of an organism may increase in size at different rates. Such processes of differential growth will change the overall shape of the body in which they occur. Processes of this kind take place very commonly in animals, particularly in the later stages of development. In animals such growth processes are greatly influenced by a variety of hormones. It is probable that factors internal to individual cells also always play a role. Although differential growth may produce striking alterations in the general shape of organisms, these effects should probably be considered as somewhat superficial, since they only modify a basic pattern laid down by other processes. In a plant, for instance, the fundamental pattern is determined by the arrangement of the lateral buds around the central growing stem; whether these buds then grow fast or slowly relative to the stem is a secondary matter, however striking its results may be.

Differentiation

Differentiation is simply the process of becoming different. If, in connection with biological development, morphogenesis is set aside as a component for separate consideration, there are two distinct types of differentiation.

- In the first type, a part of a developing system will change in character as time passes; for instance, a part of the mesoderm, starting as embryonic cells with little internal features, gradually develops striated myofilaments, and with a lapse of time develops into a fully formed muscle fibre.
- In the second type, space rather than time is involved; for instance, other cells within the same mass of embryonic mesoderm may start to lay down an external matrix around them and eventually develop into cartilage.

In development, differentiation in time involves the production of the characteristic features of the adult tissues, and is referred to as *histogenesis*. Differentiation in space involves an initially similar (homogeneous) mass of tissue becoming separated into different regions and is referred to as regionalization.

Histogenesis involves the synthesis of a number of new protein species according to an appropriate timetable. The most easily characterized are those proteins formed in a relatively late stage of histogenesis, such as myosin and actin in muscle cells. The synthesis of proteins is under the control of genes, and the problem of histogenesis essentially reduces to that of the genetic mechanisms that direct protein synthesis.

Regionalization is concerned with the appearance of differences between various parts of what is at first a homogeneous, or nearly homogeneous, mass. It is a prelude to histogenesis, which then proceeds in various directions in the different regions so demarcated. The processes by which the different regions acquire distinct contrasting characteristics must be related to some of the processes discussed under morphogenesis. Unlike morphogenesis, regionalization need not involve any change in the overall spatial shape of the tissues undergoing it. Regionalization falls rather into the type of process for which field theories have been invoked.

Growth gradient

Cells could have their position specified by a variety of mechanisms. The simplest is based on a gradient of some substance. If the concentration of some chemical decreases from one end of a line of cells to the other. Then the concentration of that chemical in any cell along the line effectively specifies the position of the cell with respect to the boundary (Fig 3). A chemical whose concentration varies and which is involved in pattern formation, is called a morphogen.

Morphogens are cytoplasmic contents that include m-RNA, cytoplasmic proteins etc. that create concentration gradient as a result cell attains its relative position, and differentiation in respect of this substance. This is illustrated in Fig 3.

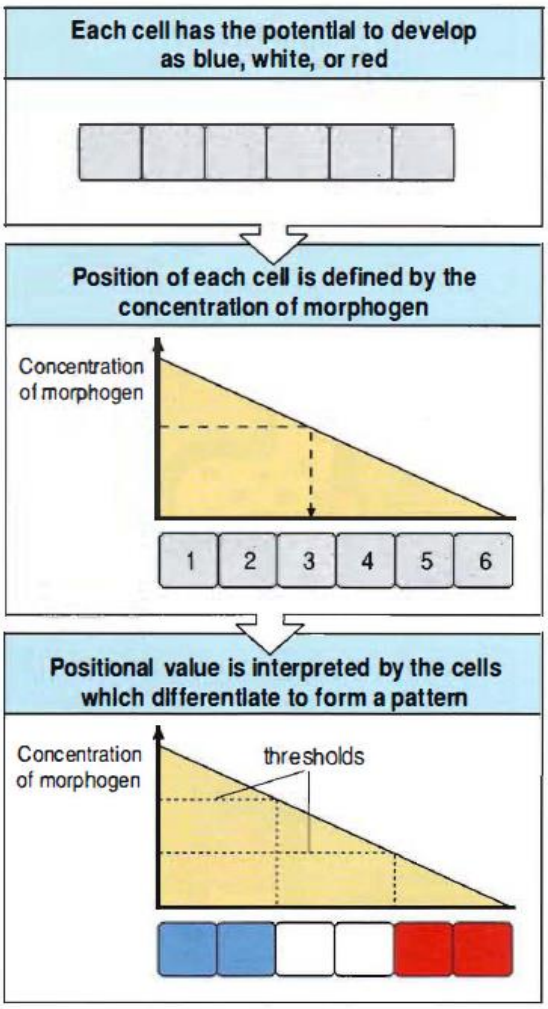

Fig 3: *The French flag model of pattern formation*. Each cell in a line of cells has the potential to develop as blue, white or red. The line of cells is exposed to a concentration gradient of some substance and each cell acquires a positional value defined by the concentration at that point. Each

cell then interprets the positional value it has acquired and differentiates into blue, white or red; according to a predetermined genetic program thus forming the French flag pattern. Substances that can direct the development of cells in this way are known as morphogens. The basic requirements of such a system are that the concentration of substance at either end of the gradient must remain different from each other but constant; thus fixing boundaries to the system. Each cell must also contain the necessary information to interpret the positional values. Interpretation of the positional value is based upon different threshold responses to different concentrations of morphogen.

Nature of action of morphogenetic gradients

- Cells usually respond to morphogen gradient by activating / repressing genes.
- Diversification of cell types and structures occur like
    - Mesoderm differentiation.
    - Somite formation.
    - Neural induction.
    - Axis formation.
    - Limb differentiation.

Mesoderm differentiation

Several of the proteins identified as possible mesoderm-patterning agents in *Xenopus*, such as the nodal-related proteins, are indeed expressed in a graded fashion. Just how gradients are set up with the necessary precision is not clear. Simple diffusion of a morphogen may play a part but more complex cellular processes are likely to be involved.

Experiments with activin provide an example of how a diffusible protein could pattern a tissue by turning on particular genes at specific threshold concentrations. Although activin itself may not be responsible for mesoderm patterning in this way in vivo, animal cap cells from a *Xenopus* blastula respond to increasing doses of activin by activation of different genes at different threshold concentrations. Increasing activin concentration by as little as l 1fz-fold results in a dramatic alteration in the pattern of marker proteins expressed and in the tissues that differentiate. For example, this small increase causes a change from homogeneous formation of muscle to formation of notochord. Increasing concentrations of activin can specify several different cell states that correspond to the different regions along the dorso-ventral axis. At the lowest concentrations of activin, only epidermis develops. Then, as the concentration increases. *Brachyury* is expressed together with muscle-specific genes such as that for actin. With a further increase in activin, *goosecoid* is expressed, and this corresponds to the dorsal-most region of the mesoderm-the organizer (Fig 4). Similar result can be obtained by injecting increasing quantities of activin mRNA.

A characteristic feature of the vertebrate body plan is a segmented body axis, most clearly seen in the skeleton. Segmentation is initiated very early in the developing embryo through the formation of segments called somites, which later give rise to vertebrae and skeletal muscle, as well as to some dermis

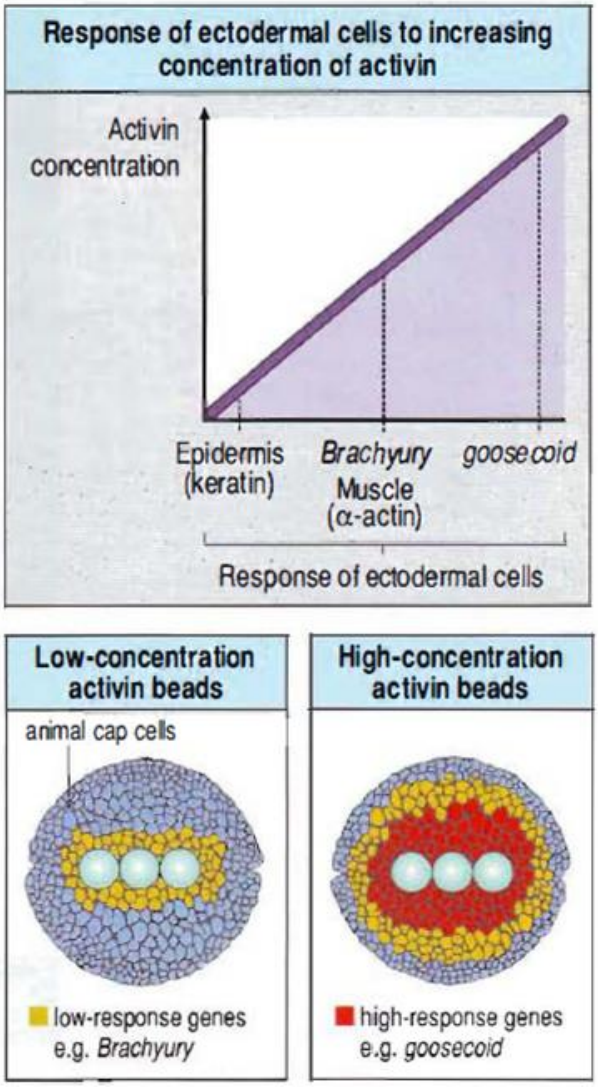

Fig 4: Graded responses of early *Xenopus* tissue to increasing concentrations of activin. When animal cap cells are treated with increasing concentrations of activing, particular genes are activated at specific concentrations. As shown in the top panel. At intermediate concentrations of activing, *Brachyury* is induced, whereas *goosecoid*, which is typical of the organizer region is only induced at high concentrations. If beads releasing a low concentration of activin are placed in the center of a mass of animal cap cells (lower left panel). Expression of low response genes such as *Brachyury* is induced immediately around the beads. With a high concentration of activin in the beads (lower right panel), goosecoid and other high-response genes are now expressed around the beads and the low-response genes farther away.

Types of cell specifications achieved by morphogens

In normal embryonic and post embryonic development, 3 types of specification occur

- **Autonomous specification**
  - Specification is achieved by differential acquisition of certain cytoplasmic molecules present in the egg.
  - Mosaic pattern of embryogenesis is found, cells cannot change their fate if a blastomere is lost from early stage of embryonic development.

- Invariant cleavage produce the same lineage in each embryo.
- Found in most of the invertebrates.

➢ **Conditional specification**

- It is done by interaction of cells. Relative position is important.
- Regulative pattern of embryogenesis is found.
- Variable cleavage produce no invariant fate assigned to cells.
- Characteristics of all vertebrates.

➢ **Syncytial specification**

- It is achieved by interaction between cytoplasmic regions prior to cellularization of blastoderm.
- Variable cleavage produce no rigid cell fates.
- Found in insects

Probable questions:

1. Discuss the growth in cell.
2. Discuss the growth in plants.
3. What is regeneration?
4. Discuss the growth pattern in tumour.
5. What do you mean by compensatory growth?
6. Elaborate different types of development.
7. Discuss about growth regulating factors.
8. Discuss briefly about relative growth.
9. Describe the types of cell specifications achieved by morphogens.
10. What do you mean by morphogen?
11. What do you mean by syncytial specification?

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Unit IV

Regeneration: origin of regenerating cells and their potentialities, Field action in regeneration

Objective: In this unit you will know about origin of regenerating cells and their potentialities, Field action in regeneration

Introduction

Regeneration can be defined as intrinsic ability of living organisms to regrow damaged or lost parts of the body or to reconstitute the whole body from a small fragment during the post embryonic life. It is a developmental process that involves growth, morphogenesis and differentiation.

Capacity of regeneration varies extensively in different animal groups. Regenerating capacity is very high among the protozoans, sponges and coelenterates. In sponges, entire body can be reconstructed from isolated body cells. Similarly, in Hydra and Planaria, small fragments of the body can give rise to a whole organism. When a hydra or a planaria is cut into many pieces, each individual part can be regenerated into a whole organism.

In most of animals, only certain parts of body show regeneration. For example, annelids like earthworms are able to regenerate some segments, removed from the anterior and posterior ends of the body. Some molluscs can regenerate only the eyes and heads. Many arthropods (Spiders) can regenerate limbs only. Regeneration power is well marked in amphibians like salamanders, Newts and their Axolotl larva. They can regenerate limbs, tail, external gills, jaws, parts of eyes like lens and retina. In mammal, regeneration is restricted to tissue only. Skin and Skeletal tissues possess the great power of regeneration. Animals with no power of regeneration at all includes nematodes (e.g. *Caenorhabditis elegans*) and rotifers.

Types of Regeneration

Physiological Regeneration

There is a constant loss of many kinds of cells due to wear and tear caused by day-to-day activities. The replacement of these cells is known as physiological regeneration.
Example:

- Replacement of R.B.C's: The worn out R.B.C's are deposited in the spleen and new R.B.C's regularly produced from the bone marrow cells, since the life span of R.B.C's is only 120days.
- Replacement of Epidermal Cells of the Skin: The cells from the outer layers of epidermis are regularly peeled off by wear and tear. These are constantly being replaced by new cells added by the epidermis layer of the skin.

Reparative Regeneration

This is the replacement of lost parts or repair of damaged body organs. In this type of regeneration, wound is repaired or closed by the expansion of the adjoining epidermis over the wound.
Example:

- Regeneration of limbs in salamanders
- Regeneration of lost tail in lizard
- Healing of wound

- Replacement of damaged cells.

Autotomy

In some animals like starfish, some part of the body is broken off on being threatened by a predator. This phenomenon of self-mutilation of the body is called autotomy
Example:

- Crabs break off their leg on approaching of the enemy
- Holothurians throw off their internal viscera
- Starfish breaks off an arm

Types of Regeneration based on Cellular Mechanism

Based on cellular mechanisms regeneration can be of two types:

1) Morphallaxis

In this type, regeneration occurs mainly by the remodelling of existing tissues and the reestablishment of boundaries, thus involving very little new growth. As a result, the regenerated individual is much smaller initially. It subsequently increases its size and becomes normal after feeding. This type of regeneration is known as *morphallaxis* or *morphallactic regeneration*.

Example: Regeneration of hydra from a small fragment of its body.

2) Epimorphosis

In this type, regeneration involves dedifferentiation of adult structures in order to form an undifferentiated mass of cells. They are highly proliferating and accumulate under the epidermis, which has already expanded. Within two days, bulge transforms into a conical hump. This lump of dedifferentiated cells along with the epidermal covering is called regeneration bud or regeneration blastema. The dedifferential cells continue to proliferate and finally redifferentiate to form a rudiment of the limb. The rudiment eventually transforms into a limb. This type of regeneration is known as epimorphosis or epimorphic regeneration.

Example: Limb regeneration in amphibians.

Origin of regenerating cells

Regenerating cells are a mass of unspecified cells or uncommitted cells but mitotically active long term reserved cells. During cells or tissue damaging, regenerating cells are specified and re-differentiated to regain their original fate to restore the original structures that have been lost. As well as regenerating cells are also involved in repatterning of existing tissue, regulated by genetic induction factors.

In biology, regeneration is the process of renewal, restoration, and growth that makes genomes, cells, organisms. Every species is capable of regeneration, from bacteria to humans. Regeneration in biology, however, mainly refers to the morphogenic processes that characterize the phenotypic plasticity of traits allowing multi-cellular organisms to repair and maintain the integrity of their physiological and morphological states. Regeneration is different from reproduction.

Cellular molecular fundamentals

Pattern formation in the morphogenesis of an animal is regulated by genetic induction factors that put cells to work after damage has occurred. Neural cells, for example, express growth-associated proteins, such as GAP-43, tubulin, actin, an array of novel neuropeptides, and cytokines that induce a

cellular physiological response to regenerate from the damage. Many of the genes that are involved in the original development of tissues are reinitialized during the regenerative process. Cells in the primordia of zebrafish fins, for example, express four genes from the homeobox *msx* family during development and regeneration.

Potentialities of regenerating cells

Potentialities indicate ultimate fate of regenerating cells or actual state of commitment of cells to particular cell lineage. Virtually no group of organisms lacks the ability to regenerate something. This process, however, is developed to a remarkable degree in lower organisms, such as protists and plants, and even in many invertebrate animals such as earthworms and starfishes. Regeneration is much more restricted in higher organisms such as mammals, in which it is probably incompatible with the evolution of other body features of greater survival value to these complex animals.

Protozoans

Most single-celled, animal-like protists regenerate very well. If part of the cell fluid, or cytoplasm, is removed from *Amoeba*, it is readily replaced. A similar process occurs in other protozoans, such as flagellates and ciliates. In each case, however, regeneration occurs only from that fragment of the cell containing the nucleus. Amputated parts that lack a nucleus cannot survive. In some ciliates, such as *Blepharisma* or *Stentor*, the nucleus may be elongated or shaped like a string of beads. If either of these organisms is cut in two so that each fragment retains part of the elongated nucleus, each half proceeds to grow back what it lacks, giving rise to a complete organism in less than six hours. The way in which such a bisected protozoan regenerates is almost identical with the way it reproduces by ordinary division. Even a very tiny fragment of the whole organism can regenerate itself, provided it contains some nuclear material to determine what is supposed to be regenerated.

Invertebrates

Coelenterates

The vast majority of research on coelenterates has been focussed on hydras and some of the colonial hydroids. If a hydra is cut in half, the head end reconstitutes a new foot, while the basal portion regenerates a new hydranth with mouth and tentacles (Fig 1).

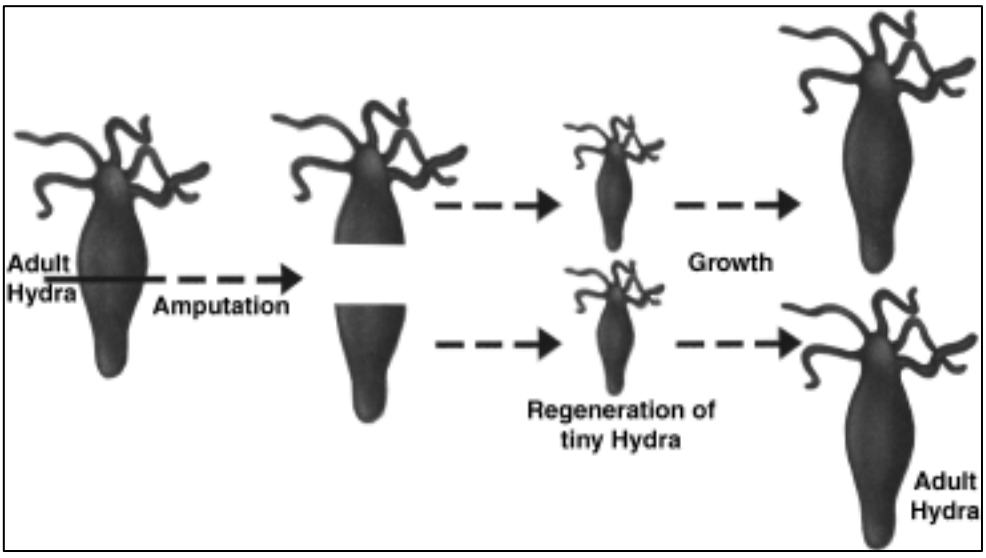

Fig 1: Regeneration of Hydra

This seemingly straightforward process is deceptively simple. From tiny fragments of the organism whole animals can be reconstituted. Even if a hydra is minced and the pieces scrambled, the fragments grow together and reorganize themselves into a complete whole. The indestructibility of the hydra may well be attributed to the fact that even the intact animal is constantly regenerating itself. Just below the mouth is a growth zone from which cells migrate into the tentacles and to the foot where they eventually die. Hence, the hydra is in a ceaseless state of turnover, with the loss of cells at the foot and at the tips of the tentacles being balanced by the production of new ones in the growth zone. If such an animal is X-rayed, the proliferation of new cells is inhibited and the hydra gradually shrinks and eventually dies owing to the inexorable demise of cells and the inability to replace them.

In colonial hydroids, such as *Tubularia*, there is a series of branching stems, each of which bears a hydranth on its end. If these hydranths are amputated they grow back within a few days. In fact, the organism normally sheds its hydranths from time to time and regenerates new ones naturally.

Flatworms

Planarian flatworms are well-known for their ability to regenerate heads and tails from cut ends. In the case of head regeneration, some blastema cells become brain tissues, others develop into the eyes, and still others differentiate as muscle or intestine. In a week or so, the new head functions almost as well as the original.

The blastema that normally gives rise to a single head is, under certain circumstances, even capable of becoming two heads if the stump of a decapitated flatworm is divided in two by a longitudinal cut. Each of the two halves then gives rise to a complete head. Thus, each blastema develops into an entire structure regardless of its size or position in relation to the rest of the animal.

In the case of flatworms there is still considerable disagreement concerning the origins of the blastema. Some investigators contend that it is derived from neoblasts, undifferentiated reserve cells scattered throughout the body. Others claim that there are no such reserve cells and that the blastema develops from formerly specialized cells near the wound that dedifferentiate to give rise to the blastema cells. Whatever their source, the cells of the blastema are capable of becoming many different things depending upon their location.

Regeneration in flatworms occurs in a stepwise fashion. The first tissue to differentiate is the brain, which induces the development of eyes. Once the head has formed, it in turn stimulates the production of the pharynx. The latter then induces the development of reproductive organs farther back. Thus, each part is necessary for the successful development of those to come after it; conversely, each part inhibits the production of more of itself. If decapitated flatworms are exposed to extracts of heads, the regeneration of their own heads is prevented. Such a complex interplay of stimulators and inhibitors is responsible for the successful regeneration of an integrated morphological structure.

Regeneration in Dugesia:

Dugesia and some related planarians possess the remarkable power of regeneration. If an animal is cut transversely into two halves (Fig 2) the anterior half containing head can easily regenerate the posterior part including a tail and a full animal can be developed.

Again the posterior half can develop a new head anteriorly and a new planarian can be developed by making up the lost parts of the individual. Regeneration can be effected by the formative cells of parenchyma.

The regeneration parts of the animal also possess the linear polarity because the anterior part always contains head and posterior part contains the tail. If the anterior end of *Dugesia* is cut longitudinally

along the mid-line (Fig 2) each part of the head will develop into a complete head, and several longitudinal pieces of the head will develop into several complete heads, thus shows the lateral polarity.

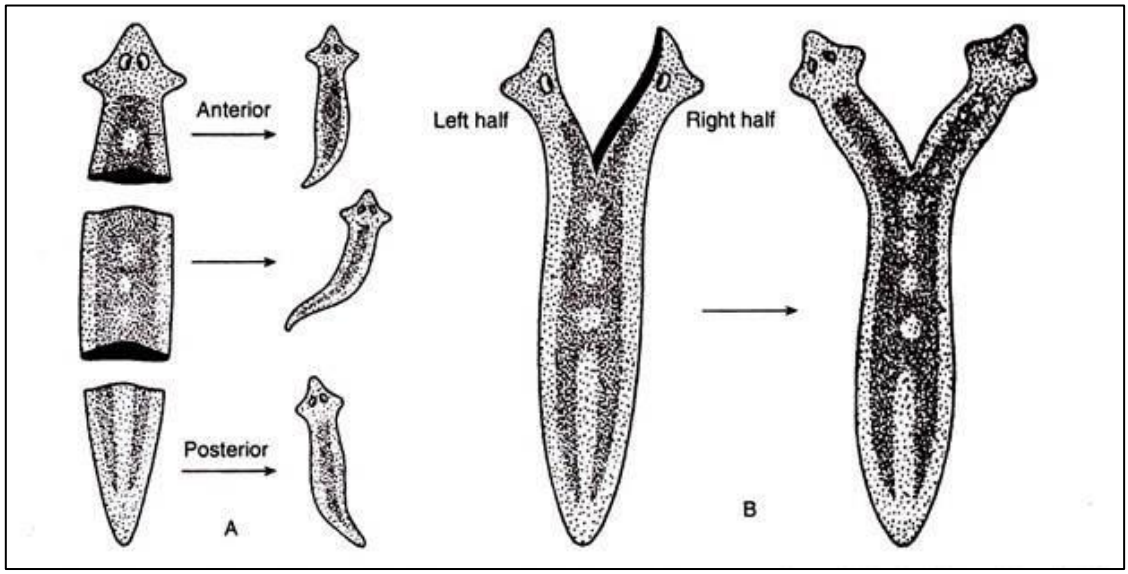

Fig 2: Regeneration in *Dugasia*. A. Excised through transverse plane B. Excised through longitudinal plane

Regeneration is effected by two processes:
(i) Epimorphosis and
(ii) Morphollaxis.

Epimorphosis is a process in which the lost parts of the planarians are formed and morphollaxis is related to the adjustment and function of the original parts with the regenerated parts.
The power of regeneration is maximum in the head region and gradually decreases towards the tail region. This power of regeneration is related to the metabolic rate of the different parts of the body. The metabolic rate is highest in the anterior region and lowest in the tail or posterior region. This variation of metabolic rate with the regenerative power is called the axial gradient.

Annelids

The segmented worms exhibit variable degrees of regeneration. The leeches, as already noted, are wholly lacking in the ability to replace lost segments, whereas the earthworms and various marine annelids (polychaetes) can often regenerate forward and backward. The expression of such regenerative capacities depends very much on the level of amputation. Anteriorly directed regeneration usually occurs best from cuts made through the front end of the worm, with little or no growth taking place from progressively more posterior bisections. Posteriorly directed regeneration is generally more common and extensive. Some species of worms replace the same number of segments as were lost. Hypomeric regeneration, in which fewer segments are produced than were removed, is more common, however.

Anterior regeneration depends upon the presence of the central nerve cord. If this is cut or deflected from the wound surface, little or no forward regeneration may take place. Posterior regeneration requires the presence of the intestine, removal of which precludes the formation of hind segments. Thus, it would seem that no head will regenerate without a central nervous system, nor a tail without an opening.

Arthropods

Many insects and crustaceans regenerate legs, claws, or antennas with apparent ease. When insect legs regenerate, the new growth is not visible externally because it develops within the next proximal segment in the stump. Not until the following molt is it released from its confinement to unfold as a fully developed leg only slightly smaller than the original. In the case of crabs, regenerating legs bulge outward from the amputation stump. They are curled up within a cuticular sheath, not to be extended until the sheath is moulted. Lobsters and crayfish regenerate claws and legs in a straightforward manner as direct outgrowths from the stumps. As in other crustaceans, however, these regenerates lie immobile within an enveloping cuticle and do not become functional until their sheath is shed at the next molt.

In all arthropods regeneration is associated with moulting, and therefore takes place only during larval or young stages. Most insects do not initiate leg regeneration unless there remains ample time prior to the next scheduled molt for the new leg to complete its development. If amputation is performed too late in the inter-moult period, the onset of regeneration is delayed until after shedding; the regenerate then does not appear until the second molt. Metamorphosis into the adult stage marks the end of moulting in insects, and adults accordingly do not regenerate amputated appendages.

Crustaceans often tend to molt and grow throughout life. They therefore never lose the ability to grow back missing appendages. When a leg is lost, a new outgrowth appears even if the animal is not destined to molt for many months. Following a period of basal growth, during which a diminutive limb is produced, the regenerated part eventually ceases to elongate. Not until a few weeks before the next molt does it resume growth and complete its development, triggered by the hormones that induce moulting.

Echinoderms

Tissue regeneration is widespread among echinoderms and has been well documented in starfish *(Asteroidea)*, sea cucumbers *(Holothuroidea)*, and sea urchins *(Echinoidea)*. Appendage regeneration in echinoderms has been studied since at least the 19th century. In addition to appendages, some species can regenerate internal organs and parts of their central nervous system. In response to injury starfish can autotomize damaged appendages. Autotomy is the self-amputation of a body part, usually an appendage. Depending on severity, starfish will then go through a four-week process where the appendage will be regenerated. Some species must retain mouth cells in order to regenerate an appendage, due to the need for energy. The first organs to regenerate, in all species documented to date, are associated with the digestive tract. Thus, most knowledge about visceral regeneration in holothurians concerns this system.

Fig 3: Sun flower sea star regenerates its arms

Vertebrates

Fishes

Many different parts of the fish's body will grow back. Plucked scales are promptly replaced by new ones, and amputated gill filaments can regenerate easily. The "whiskers," or taste barbels, of the catfish grow back as perfect replicas of the originals. The most conspicuous regenerating structures in fishes, however, are the fins. When any of these are amputated, new fins grow out from the stumps and soon restore everything that was missing. Even the coloured stripes or spots that adorn some fins are reconstituted by new pigment cells that repopulate the regenerated part. Fin regeneration depends on an adequate nerve supply. If the nerves are cut leading into the fin, regeneration of neither the amputated fin nor excised pieces of the bony fin rays can take place.

Amphibians

Salamanders are remarkable for their ability to regenerate limbs. Larval frogs, or tadpoles, also possess this ability, but usually lose it when they become frogs. It is not known why frog legs do not regenerate, and under appropriate stimuli they can be induced to do so.

Tadpoles and salamanders can replace amputated tails. Tadpole tails have a stiff rod called the notochord for support, whereas salamanders possess a backbone, composed of vertebrae. Both tails contain a spinal cord. When the salamander regenerates its tail, the spinal cord grows back and segmental nerve-cell clusters (ganglia) differentiate. Tadpoles also regenerate their spinal cords, but not the associated ganglia. If the spinal cord is removed or destroyed in the salamander, no tail regeneration occurs; if it is removed from the tadpole tail, however, regeneration can proceed without it.

Fig 4: Dwarf yellow headed Gecko with regenerating tail

Regeneration of a limb of a newt or salamander

Newt/salamander has very high power of regenerating their lost limb by the process of restorative regeneration. It involves the following steps (Fig 5).

(1) **Wound healing:** The epidermal cells from the edges of the cut migrate and spread over the exposed surface. This is known as wound healing.

(2) **Blastema formation:** A few days after the healing of the cut, the undifferentiated cells accumulate inside the epidermis. Due to this cellular aggregation, a stumpy outgrowth or bulge is formed. This is known as regeneration bud or blastema.

(3) **Redifferentiation and morphogenesis:** The blastema develops rudiments of digits by indentation at the free edge. These grow out into new digits.

(4) **Growth:** The regenerated limb increases till it attains the size of a normal limb.

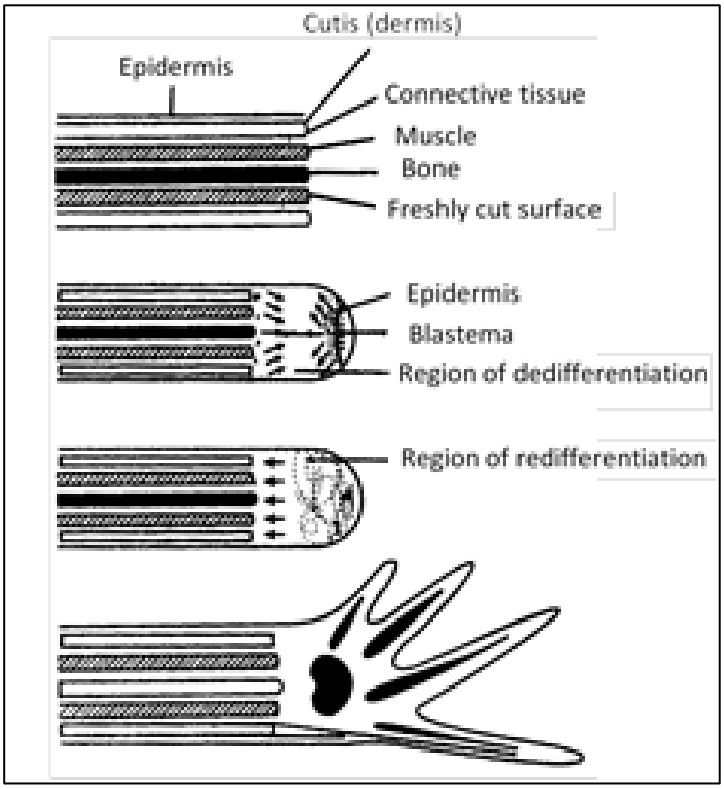

Fig 5: Changes during the regeneration of limbs of Salamander

Reptiles

Lizards also regenerate their tails, especially in those species that have evolved a mechanism for breaking off the original tail when it is grasped by an enemy. When the lizard tail regenerates, however, it does not replace the segmented vertebrae. Instead, there develops a long tapering cartilaginous tube within which the spinal cord is located and outside of which are segmented muscles. The spinal cord of the lizard tail is necessary for regeneration, but the regenerated tail does not reproduce the ganglia that are normally associated with it. Occasionally, a side tail may be produced if the original tail is broken but not lost.

Birds

Regeneration of amputated appendages in birds is not known to occur; however, they do replace their feathers as a matter of course. While most species shed and regenerate feathers one at a time so as not to be grounded, flightless birds, such as penguins, may molt them all at once. Male puffins cast off their colourful beaks after the mating season, but grow new ones the following year. In like manner, the dorsal keel on the upper beaks of male pelicans is shed and replaced annually.

Mammals

Although mammals are incapable of regenerating limbs and tails, there are a few exceptional cases in which lost tissues are in fact regenerated. Not the least of these cases is the annual replacement of antlers in deer. These remarkable structures, which normally grow on the heads of male deer,

consist of an inner core of bone enveloped by a layer of skin and nourished by a copious blood supply. During the growing season the antlers elongate by the proliferation of tissues at their growing tips. The rate of growth in some of the larger species may surpass one centimetre (0.39 inch) per day; the maximum rate of growth recorded for the elk (*Cervus elaphus canadensis*) is 2.75 centimetres (1.05 inches) per day. When the antlers have reached their full extent, the blood supply is constricted, and the skin, or velvet, peels off, thus revealing the hard, dead, bony antlers produced by the male deer in time for the autumn mating season. The regeneration of elk antlers spans about seven months. The following spring, the old antlers are shed and new ones grow to replace them.

Still another example of mammalian regeneration occurs in the case of the rabbit's ear. When a hole is punched through the external ear of the rabbit, tissue grows in from around the edges until the original opening is reduced or obliterated altogether. This regeneration is achieved by the production of new skin and cartilage from the margins of the original hole. A similar phenomenon occurs in the case of the bat's wing membrane.

Regulation of regeneration

There are certain prerequisites without which regeneration cannot occur. First and foremost, there must be a wound, although the original appendage need not have been lost in the process. Second, there must be a source of blastema cells derived from remnants of the original structure or an associated one. Finally, regeneration must be stimulated by some external force. The stimuli often involve the nervous system. An adequate nerve supply is required for the regeneration of fish fins, taste barbels, and amphibian limbs. In the case of many tail regenerations, the spinal cord provides the necessary stimulus. Lens regeneration in salamander eyes depends upon the presence of a retina. Arthropod appendages regenerate in the presence of moulting hormones. Protozoan regeneration requires the presence of a nucleus. In case after case, regeneration depends on more than a healed wound and a source of blastema cells. It is often triggered by some physiological stimulus originating elsewhere in the body, a stimulus invariably associated with the very function of the structure to be regenerated. The conclusion is inescapable that regeneration is primarily the recovery of deficient functions rather than simply the replacement of lost structures.

The imperative of need is of further importance in suppressing excess regeneration. To be able to regenerate is to run the risk of regenerating too much or too often. If regeneration did not depend upon a physiological stimulus, such as those mediated by nerves or hormones, there would be no reason why simple wounds should not sprout whole new appendages.

It is not known why regeneration fails to occur in many cases, as in the legs of frogs or the limbs and tails of mammals. The nerve supply might be inadequate, for when the number of nerves is artificially increased, regeneration is sometimes induced. This cannot be the whole answer, however, because not all appendages depend on nerves for their regeneration; newt jaws, salamander gills, and deer antlers do not require nerves to regenerate.

Probable questions:

1. Define regeneration.
2. Describe the types of regeneration.
3. Define autotomy.
4. What do you mean by morphallaxis? Give example.
5. What do you mean by epimorphosis? Give example.
6. Discuss in details about the potentialities of regenerating cells in echinoderms.
7. Discuss in details about the potentialities of regenerating cells in arthopods.
8. Discuss in details about the potentialities of regenerating cells in vertebrates.
9. Discuss in details about the potentialities of regenerating cells in invertebrates.
10. Describe the Regeneration in Dugesia with diagram.
11. Describe the regeneration of a limb of a newt or salamander with proper diagram.
12. Discuss on regulation of regeneration.

Suggested readings:

1. Gilbert, S. F. (2010). Developmental Biology, IX Edition, Sinauer Associates, Inc.,

Publishers, Sunderland, Massachusetts, USA

2. Slack JMW (2006). Essential Developmental Biology. 2nd Edn. Blackwell Pub.

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4. Verma and Agarwal. Developmental Biology. S. Chand Pub. New Delhi

Unit V

Proteins during development: Lens crystalline: Classification, ontogeny of crystalline in fish, chick and mammals

Objective: In this unit you will know about Proteins during development: Lens crystalline: Classification, ontogeny of crystalline in fish, chick and mammals

Introduction

The structural proteins in the eye lens that are responsible for the refractive properties of this tissue are called the crystalline. The crystalline makes up more than 90% of the total dry mass of the lens. In the mammalian lens there are, in general, three classes of crystalline: α, β, γ. Each crystalline class constitutes about one-third of the total mass. There are two α-crystalline genes; αA and αB. In humans, for example, the αA gene encodes for a 173 amino acid residues polypeptide and the αB gene encodes for a 175 amino acid residues polypeptide. There is a 57% sequence homology between αA and αB. In all vertebrate lenses α-crystalline is found as a heterogeneous multimeric complex with a molecular weight distribution ranging from 300 000 to over 1 million. The average molecular weight of α-crystalline in mammalian lenses is estimated at 600 000 to 900 000. Thus each native α-crystalline complex is made up of 30 to 45 subunits. In these native multimeric complexes the ratio of αA to αB is approximately 3: l.

Until the late 1980s it was believed that the crystallines were lens-specific proteins. However, it was found that αB-crystalline is essentially a ubiquitous protein and is a bona fide member of the small heat-shock protein family. αA-crystalline is found mainly in the lens. It has been shown in recent years that αB-crystalline is overexpressed in various neurodegenerative diseases. It is also elevated in the ischaemic heart and in other biological systems where stress is introduced.

In 1992, it was shown that α-crystalline has chaperone-like properties being capable of binding to unfolded or denatured proteins and suppressing non-specific aggregation. The finding that a-crystalline possesses chaperone like properties suggests that it may have a functional role in the lens, in addition to its structural role as a major refractive element. In the lens there is no protein turnover, repair mechanisms are minimal, post-translational alteration and protein unfolding continue throughout life, and transparency has to be maintained for decades. The chaperone-like properties of α-crystalline seem to be most suitable for a unique tissue such as the eye lens where proteins, especially in the lens centre, are as old as the organism.

Classification

Crystallines from a vertebrate eye lens are classified into three types: α, β and γ crystallines. These distinctions are based on the order in which they elute from a gel filtration chromatography column. These are also called ubiquitous crystallines. β and γ crystallines are similar in sequence, structure and domains topology, and thus have been grouped together as a protein superfamily called βγ-Crystallines. The α-crystalline superfamily and βγ-crystallines compose the major superfamily of proteins present in the crystalline lens.

In addition to these crystallines there are other taxon-specific crystallines which are only found in the lens of some organisms; these include δ, ε (epsilon), τ (tau), and ι (iota)-crystallines. For example, α, β, and δ crystallines are found in avian and reptilian lenses, and the α, β, and γ families are found in the lenses of all other vertebrates.

α Crystalline:

α-crystalline is a major lens protein, comprising up to 40% of total lens proteins, where its structural function is to assist in maintaining the proper refractive index in the lens. It occurs as large aggregates, comprising two types of related subunits (A and B) that are highly similar to the small (15-30kDa) heat shock proteins (HSPs), particularly in their C-terminal halves. The relationship between these families is one of classic gene duplication and divergence, from the small HSP family, allowing adaptation to novel functions. Divergence probably occurred prior to evolution of the eye lens, α-crystalline being found in small amounts in tissues outside the lens.

α-crystalline has chaperone-like properties including the ability to prevent the precipitation of denatured proteins and to increase cellular tolerance to stress. It has been suggested that these functions are important for the maintenance of lens transparency and the prevention of cataracts. This is supported by the observation that α-crystalline mutations show an association with cataract formation. The N-terminal domain of α-crystalline is not necessary for dimerization or chaperone activity, but appears to be required for the formation of higher order aggregates.

β and γ Crystalline:

β and γ - crystalline form a separate family. Structurally, β and γ crystallines are composed of two similar domains which, in turn, are each composed of two similar motifs with the two domains connected by a short connecting peptide. Each motif, which is about 40 amino acid residues long, is folded in a distinctive Greek key pattern. However, β crystalline is an oligomer, composed of a complex group of molecules, whereas gamma crystalline is a simpler monomer

Ontogeny of Lens Crystalline

The tremendous evolutionary advantages conferred by the ability to respond to light are evident in the success of species from the unicellular, with simple eyespots, to vertebrates with image forming eyes. In the animal kingdom, the six phyla (out of 35) that are most widespread and numerous are those that have image forming eyes while many others have light sensing systems. In all eyes, light is absorbed by related members of the opsin superfamily arrayed in either rhabdomeric or ciliary photoreceptor cells that transduce the optical signal through distinct mechanisms. Beyond this basic level of light sensitivity, the structures and optics of eyes are extremely diverse. Eyes can be single or compound, gathering, and directing light onto the photoreceptors of the retina with pinholes, lenses, cylinders, or mirrors. Vertebrates use a camera eye with a cellular lens situated behind a curved cornea. In fish, underwater, the lens alone provides almost all the focusing power, while in terrestrial species, in air, the cornea provides most focusing power and the lens is mainly used for fine control of image formation.

Ontogeny of crystalline in vertebrate

The vertebrate lens is derived embryologically from an invaginated ectodermal epithelium, the lens vesicle, and grows throughout life by the orderly proliferation and differentiation of epithelial cells into layers of extremely elongated fiber cells. Cell organization is important for lens transparency and focusing, but most of the refractive power of the lens is conferred by high concentrations of proteins, with any highly abundant protein being designated a crystalline.

There are two unique features to the vertebrate eye lens.

- First, there is a very high concentration of structural proteins (crystallines), reaching values in excess of 500 mg/ml. The high protein concentration is needed to obtain the necessary refractive index for the lens.
- Second, in the lens there is continued growth throughout life and lack of protein turnover in the terminally differentiated lens fibre cells. These features are the consequence of the 'design' and the programmed development and growth of the eye lens.

It is well established that many of the lens crystallines in the animal kingdom were 'recruited' from existing enzymes to serve an additional role as a structural protein. The dual function of a protein that can serve both as a structural protein and as an enzyme led to the recent concept of 'gene-sharing. This concept simply means that the same gene acquires an additional function without duplication. α-crystalline is a clear example of gene sharing. αB-crystalline, which is a *bona fide* small heat shock protein, can be found, for example, in the embryonic mouse heart before the eye is formed. αA-crystalline, on the other hand, is thought to arise by gene duplication with a more specific role as a lens crystalline.

The chaperone-like properties of the **α-crystallines** may be the reason why these particular proteins were 'recruited' to serve the lens. If one considers, for example, a 60-year-old normal person, the proteins in the centre of this person's lens are 60 years old! During the six decades these proteins were subjected to a variety of insults, stresses, and major post-translational modifications, causing them to unfold and denature. Denatured or unfolded proteins tend to aggregate, and protein aggregation in the lens will lead to light scattering and cataract. Since the old proteins in the centre of the lens cannot be repaired, and nor can they be catabolised and resynthesized, having a protein with chaperone-like properties in large amounts can alleviate the problems. α-crystalline, which recognises unfolded or denatured proteins, can selectively bind to them and arrest nonspecific aggregation. The evidence suggests that α-crystalline serves as a one-way 'sink' in the lens, binding and controlling the unavoidable denaturation of proteins taking place during normal ageing. Unlike chaperone systems in other cells, where the ultimate goal is to refold an unfolded protein, α-crystalline in the lens serves only as a part of a typical complex chaperone pathway. In the lens there is no way to refold the damaged old protein, so α-crystalline, by binding to them, controls and avoids non-specific aggregation. On ageing it is only β and γ-crystalline that are denaturing. It should be emphasised, however, that in these fractions there are relatively small but significant amounts of housekeeping enzymes such as glyceraldehyde-3-phosphate dehydrogenase, enolase, leucine aminopeptidase and aldehyde dehydrogenase. These enzymes are much more susceptible to denaturation than the other crystallines. It was shown recently that without the protein of α-crystallines, aggregation of minor lens components can contribute to the process of opacification.

The **β- and γ-crystallines** are not related to α-crystallins but are members of another protein superfamily of restricted phylogenetic and tissue distribution. In vertebrates, β- and γ-crystallins are highly expressed in the lens, with low levels found in some other eye tissues, particularly in different retinal cell types. In many vertebrate lineages, the optical properties of the lens have been also modified to adapt to environmental constraints by loss of some crystallins (generally γ-crystallins) and by independent recruitment of other proteins which, surprisingly, are usually well characterized enzymes.

Ontogeny of crystalline in chick

β-crystalline polypeptide, the 35 kDa and its mRNA are selectively expressed in the elongating cells of the epithelium and the fibre cells of the developing chicken lens. This 35 kDa β-crystalline polypeptide has recently been called βBl because of its high homology with bovine and rat lens βBl polypeptide. α- crystalline is detected in the epithelial cells of the lens placode before the appearance of any other lens antigens. Since it is also present in the pigment layer of the retina and iris of 72-hour-old embryos—that is, those tissues which have a capacity for regeneration of the lens— and is found in identical or partially identical form in the lenses of representative animals throughout the vertebrate series, it may be considered as an "ancient" lens protein which plays a key role in formation of the lens. β- crystalline appears at the onset of differentiation and growth of the nuclear lens fibers and is characterized by the rapid formation of 4 closely related subfractions. Since it is found in identical or partially identical form only in the lenses of birds and reptiles, but not in those of mammals, amphibians, and fishes, it may be considered a specialized protein which in the course of evolution develops along divergent lines. Its highly differentiated form and rapid formation may make it a target for teratogenic factors, such as German measles virus, which causes cataract only when it acts during growth and differentiation of the nuclear lens -fibers—that is, the time of β-crystalline synthesis. γ-crystalline—the last lens protein to arise during development—takes an intermediate position between alpha and β-crystalline in regard to its species-specific properties.

Probable questions:

1. What is lens crystalline?
2. Classify the crystallines from a vertebrate eye lens.
3. Write short note on alfa crystalline.
4. Write short note on beta and gama crystalline.
5. Describe the ontogeny of lens crystalline.
6. Describe the ontogeny of crystalline in vertebrates.
7. Describe the ontogeny of crystalline in chick.

Suggested readings:

1. Gilbert, S. F. (2010). Developmental Biology, IX Edition, Sinauer Associates, Inc.,

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2. Slack JMW (2006). Essential Developmental Biology. 2nd Edn. Blackwell Pub.

3. Schoenwolf, G.C., Bleyl, S.B., Brauer, P.R. and Francis-West, P.H. (2009). Ladesn's Human Embryology. 4th Edn. Elsevier

Unit VI
Haemoglobin: structure, heterogeny and ontogeny

Objective: In this unit you will know about structure of haemoglobin, heterogeny and ontogeny of haemoglobin.

Introduction

Hemoglobin, also spelled haemoglobin is the red colouring matter of blood which is present in the red blood cells. It is a conjugated protein consisting of heme and the protein globin. It has a molecular weight of 64,450 g/mol.

Haemoglobin is one of the two oxygen binding proteins found in vertebrates. Its function is to carry oxygen in the blood from the lungs to other tissues in the body, in order to supply the cells with the oxygen required by them for oxidative phosphorylation of foodstuffs. It contains 4 gram atoms of iron per mole in the ferrous (Fe^{++}) state. Haemoglobin is found in the blood within the erythrocytes (red blood cells). These cells essentially act as a sack for carrying haemoglobin, since mature erythrocytes lack any internal organelles (nucleus, mitochondria, etc). The other oxygen binding protein found in vertebrates is myoglobin, which stores the oxygen in the tissues of the body ready for when the tissues require it. The highest concentration of myoglobin are found in skeletal and cardiac muscle which requires large amounts of oxygen because of their need for large amounts of energy during contraction.

Mygolobin is the simpler of the two oxygen binding proteins. It is made up of 153 amino acids in a single polypeptide chain, and it was the first protein to have its three dimensional structure determined by x-ray crystallography. Within a hydrophobic crevice of the globular protein, formed by the folding of the poly peptide chain is the heme prosthetic group (shown in the picture above, the polypeptide part shown in pink, and the heme group shown in green). The heme group has a central iron atom which is essential in the binding of oxygen.

Properties of Haemoglobin:

i. The most characteristic property of haemoglobin is the ease with which it combines with oxygen and dissociates from it. 100 ml of water will absorb one-third ml of oxygen at body temperature under atmospheric pressure. But 100 ml of blood, under the same condition, will take up 20 ml of oxygen (60 times), due to the presence of haemoglobin. [About 1,200 ml of oxygen can be carried by the total amount of blood in an adult man.]

ii. One gram of haemoglobin combines at normal (standard) temperature pressure (NTP) with 1.34 ml of oxygen. This corresponds to two atoms of oxygen for each atom of iron. The compound oxyhaemoglobin gives off its full oxygen content when placed in vacuum.

iii. Oxyhaemoglobin holds its oxygen loosely which can be easily displaced by many other gases forming more stable compounds, e.g., CO, CO_2, NO, H_2S will form carboxyhaemoglobin, carbaminohaemoglobin, nitric oxide haemoglobin, sulphaemoglobin respectively.

iv. The globin part of haemoglobin directly combines with CO_2 and forms carbamino compounds.

v. Crystallisation- Haemoglobin can be easily crystallised. The form of the crystals, their solubility and the ease of crystallisation are characteristic of the species from which haemoglobin is

obtained. Most bloods, including human blood, form rhombic prisms or needles. Haemoglobins of different species are said to be immunologically distinct.

vi. The distinction lies in the globin part of the molecule and not in the haem part. It is known that the amino acid composition of the various globins (derived from haemoglobin of different species) varies considerably specially in respect to their cystine content. The haemoglobin of different species also shows different affinity for oxygen.

vii. Isoelectric pH of haemoglobin (reduced Hb) is 6.8 that of oxyhaemoglobin is 6.6.

viii. Spectroscopic Appearance– Haemoglohin (reduced Hb) gives one broad band between the Fraunhofer's lines D and E (corresponding to the wave-length-λ 559). That of oxyhaemoglobin consists of two bands between D and E. The band nearer D is called α-band (corresponding to the wave-length -λ 579). The band nearer E is broader and is called the β-band (with a corresponding wave length of λ 542).

Structure:

The structure of Haemoglobin can be classified under two headings:

a. Structure of Heme, the prosthetic group.
b. Structure of Globin, the protein part— apoprotein.

a. Structure of Heme:

i. It is an iron porphyrin. The porphyrins are cyclic compounds with "tetra pyrrole" structure (Fig 1).

ii. Four pyrrole rings called I to IV are linked through methylene bridges or methylene bridges.

iii. The outer carbon atoms, which are not linked with the methylene bridges, are numbered 1 to 8.

iv. The methylene bridges are designated as α, β, γ, δ, respectively.

v. Iron in the ferrous state is bound to the nitrogen atom of the pyrrole rings.

vi. Iron is also linked internally (5th linkage) to the nitrogen of the imidazole ring of Histidine of the polypeptide chains.

vii. The propionic acid of 6th and 7th position of heme of III and IV pyrroles are also linked to the amino acids Arg and Lys of the polypeptide chain, respectively.

The porphyrins are found in nature in which the various side chains are substituted for the 8 hydrogen atoms as numbered in the porphin nu¬cleus. The arrangement of the A and P substituents in the uroporphyrin shown here is asymmetric (in ring IV the expected order of the acetate and propi¬onate substituents is reversed).
This type of asym¬metric substitution is classified as a type III por¬phyrin. A porphyrin with a completely symmetri¬cal arrangement of the substituents is classified as a type I porphyrin. Only types I and III are found in nature and the type III series is more abundant.

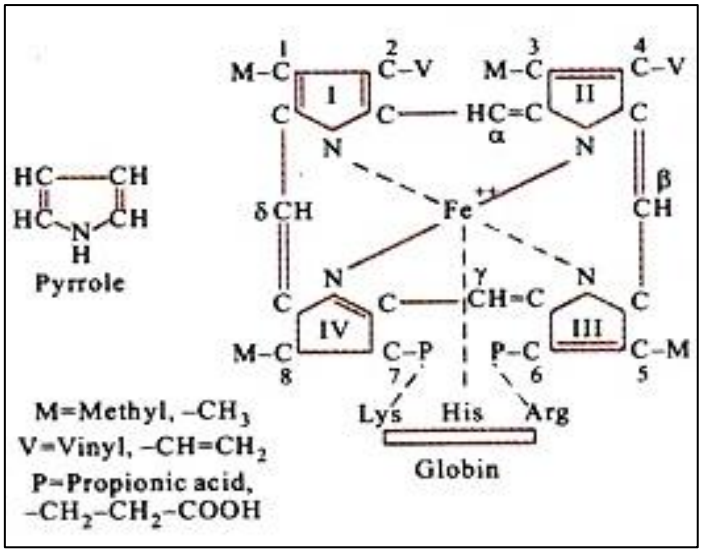

Fig 1: Structure of heme

b. Structure of Globin:

i. The globin of haemoglobin is a protein which is composed of 4 parallel layers of closely packed polypeptide chains.

ii. Two of the chains (α-chains) have identical amino acid composition of 141 amino acids. The two other chains may be two of the 4 polypeptide chains designated as β, γ, δ, and ε (epsilon). Each is having 146 amino acids.

iii. The total number of amino acids in globin is 574.

iv. α chains have Val-Leu-Ser in N terminal residues and Lys-tyr-Arg in C terminal residues.

v. β chains have Val-His-Leu in N-terminal residues and Lys-tyr-His in C-terminal residues.

vi. γ chains have Gly-His-Phe. N-terminal residues and Arg-Tyr-His in C-terminal residues.

vii. Haemoglobin molecule and its sub-units contain mostly hydrophobic amino acids internally and hydrophilic amino acids on their surfaces. So they form "'Heme pockets".

ix. In **"**heme pockets" α subunits are of size necessary for entry of O_2 molecule but the entry of O_2 molecule in β subunit is blocked by valine residue

Haemoglobin Derivatives

There are some derivatives of normal Hb that arise due to metabolic changes in the RBC.

The various haemoglobin derivatives are:

1. Oxyhemoglobin (HbO_2):

The main function of haemoglobin is to transport oxygen from the lung to the tissues. In lungs the partial pressure of oxygen is 100 mm of Hg, at this pressure haemoglobin is 95-96% saturated with oxygen. On binding with O_2 in the lungs haemoglobin is converted to oxy-haemoglobin (Hb02). O_2 is bound to heme iron.

$$Hb + O_2 \rightarrow HbO_2$$

2. Reduced Haemoglobin (HHb):

Oxy-haemoglobin moves to the tissue where the partial pressure of O_2 is 26 mm of Hg due to which oxygen is released into the tissues and in turn H^+ binds to Hb and forms reduced hemoglobin.

$$HbO_2 + H^+ \rightarrow HHb + O_2$$

3. Carbaminohaemoglobin:

Haemoglobin also binds to CO_2 in the tissues. CO_2 is bound to the α-amino group at the N-terminal end of each of the four polypeptide chains of haemoglobin to form carbaminohaemoglobin. As one CO_2 binds O_2 is released.

4. Methemoglobin:

In RBC the iron of haemoglobin is normally in ferrous (Fe^{2+}) form, but it is readily oxidized to the ferric (Fe^{3+}) form by hydrogen peroxide formed by RBC cell metabolism, to yield met-haemoglobin. Ferric iron is incapable of binding O_2 therefore the functions of haemoglobin are disturbed. Normally 1.7 to 2.4 % of total haemoglobin will be in the form of met-haemoglobin. Increase in the percent of met-haemoglobin is prevented by the peroxidase action of a naturally occurring peptide known as glutathione present in the RBC. Met-haemoglobin is dark brown in colour.

The percent of met-haemoglobin can increase if the person consumes drugs like ferricyanide, nitrite, quinines, hydroxylamine's, acetanilide and sulphonamide. Higher levels of met-haemoglobin is observed clinically in factory workers who inhale (or contact through skin) aromatic nitro and amino compounds and in patients taking large amounts of acetanilide and sulphonamides. The symptoms are cyanosis (blue skin) and dyspnoea (labored breathing).

Importance of methemoglobin:

Met-haemoglobin can be used to overcome cyanide poisoning. By injecting met-haemoglobin it combines with cyanide to form cyanomethemoglobin preventing cyanide poisoning.

5. Carboxyhaemoglobin:

Oxy-haemoglobin can bind to carbon monoxide (CO). Even normal, non- oxygenated haemoglobin can bind with CO to form carboxyhaemoglobin. [$Hb + CO \rightarrow HbCO$]. CO has got an affinity of 200 times more than that of O_2 towards Hb. Haemoglobin can bind more readily to CO than to O_2. Even if there is a little amount of CO in air, it can displace oxyHb to form carboxyHb. Due to this there will be tissue hypoxia because the oxygen binding capacity is reduced and there is also reduced O_2 releasing capacity i.e. it cannot release O_2 though it may be bounded to O_2.

City dwellers have at least 1% of carboxyhaemoglobin which can increase to 8% depending upon the pollution. Over traffic can increase carboxyHb to 40% which leads to death. Clinically such patients show cherry red colour of skin. CO poisoning can be treated if high amount of O_2 is provided continuously at high pressure, then at such high concentrations and pressure HbCO is dissociated forming HbO_2 + CO. When treatment continues for 2 hours CO is expelled out.

Types of Haemoglobin:

There are three types of haemoglobin's that are normally found in human beings, they are:

1. HbA;

Found in normal adult human beings – contains 2α and 2β chains.

2. HbA_2:

Found in some human beings and is considered normal — contains 2α and 2β chains.

3. HbF:

Foetal haemoglobin — found in growing foetus — contains 2α and 2γ chains.

Each chain is synthesized by the information obtained from the gene for haemoglobin, α chain is synthesized from a genes of haemoglobin, β chain from β genes of haemoglobin likewise y and 8 from their respective genes. There are 2 pairs of a genes but only one pair each of β, γ and δ genes.

Abnormal haemoglobin's arise due to mutation in the gene for the haemoglobin synthesis. There are about 300 abnormal haemoglobin's. Some of them are those which have defect in α genes and some are with defective β chains.

Ontogeny of haemoglobin

Biosynthesis of Porphyrins:

Heme (the iron-containing porphyrin) of hemoglobin in animals are synthesized in living cells by a common pathway:

i. The starting materials are 'active succinate' (succinyl-CoA) derived from the citric acid cycle and glycine. Pyridoxal phosphate (B_6-PO_4) is necessary to activate glycine. The product of the condensation reaction is α-amino-β-ketoadipic acid which is catalysed by the enzyme AmLev synthetase (ALA synthase).

ii. α-amino-β-ketoadipic acid is rapidly decarboxylated by the same enzyme AmLev synthetase producing δ-aminolevulinic acid (AmLev). Synthesis of aminolevulinic acid occurs in the mitochondria. The anaemia has been observed in the deficiency of vitamin $B_{(}$, or pantothenic acid.

iii. 2 mols of AmLev condense to form porphobilinogen (the first precursor of pyrrole) which is catalyzed by the enzyme δ-aminolevulinase (AmLev dehydrase).

iv. 3 mols of porphobilinogen condense first to form a tripyrrylmethane which then breaks down into a di-pyrrylmethane and a monopyrrole. The dipyrryl compounds are of two types A and B. The formation of tetrapyrrole occurs by condensation of two dipyrrylmethanes. If two of the (A) components condense, a type I porphyrin results; if one (A) and one (B) condense, a type III results.

v. The uroporphyrinogens I and III are converted to coproporphyrinogens I and III by decarboxylation being catalyzed by uroporphyrinogen decarboxylase.

vi. The coproporphyrinogen III then enters the mitochondria where it is converted to protoporphyrinogen III and then to protoporphyrin III. The enzyme coproporphyrinogen oxidase catalyzes the formation of protoporphyrinogen III. The oxidation of protoporphyrinogen to protoporphyrin is catalyzed by the enzyme protoporphyrinogen oxidase.

The enzyme coproporphyrinogen oxidase is able to act on type III coproporphyrinogen only for which type I protoporphyrin has not been identified in natural materials. In mammalian liver the reaction of conversion of coproporphyrinogen to protoporphyrin requires molecular oxygen.

vii. In the final step of heme synthesis ferrous ion (Fe^{++}) is incorporated into protoporphyrin III which is catalyzed by heme synthetase or ferrochelatase. The reaction takes place readily in the absence of enzymes but becomes rapid in presence of enzymes.

Properties of porphyrins:

1. They act both as acids (-COOH) and bases (-NH2).
2. Their isoelectric pH is 3-4.5.
3. Porphyrins are fluorescent and coloured due to presence of alternating double bonds.
4. Porphyrinogens are colourless.

Biosynthesis of heme

i. Heme is an iron porphyrin structure, synthesized in the reticuloendothelial cells (bone marrow) of adult human being. Erythropoietin produced in kidney stimulates the formation, maturation and release of erythrocytes by bone marrow.

ii. Early stage of erythrocyte cells contain porphyrin, during the course of their development, porphyrin is converted to heme by addition of iron and then to haemoglobin by addition of protein, globin. The type of porphyrin present in heme is protoporphyrin-III (also known as No. IX).

iii. It is synthesized starting from glycine and succinyl-CoA. Given below is the diagrammatic representation of biosynthesis of Heme where 'A' stands for acetyl group, 'P' stands for propyl group, 'M' for methyl group, and 'V' for venyl group (Fig 2).

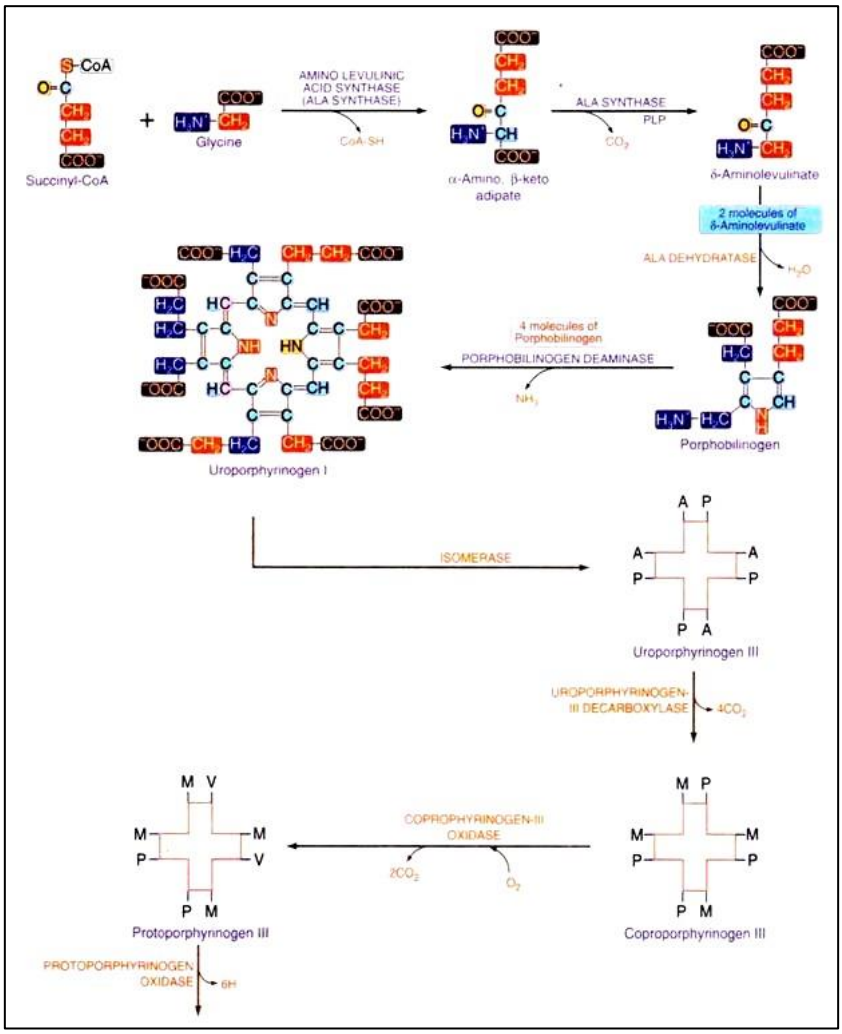

Fig 2: Diagrammatic representation of biosynthesis of Heme

Regulation of heme synthesis:

The first enzyme in this sequence i.e. ALA synthase is the key regulatory enzyme for heme synthesis which is inhibited by heme the end product of the metabolism. ALA synthase is a regulatory enzyme. Heme acts as an Apo repressor molecule and is a negative regulator for the synthesis of ALA synthase-I (heme inhibits the gene).

Functions of Haemoglobin:

1. It is essential for oxygen carriage.
2. It plays an important part in CO_2 transport.
3. It constitutes one of the important buffers of blood and helps to maintain its acid-base balance.
4. Various pigments of bile, stool, urine, etc., are formed from it.

Heterogeny of haemoglobin:

Normal adult hemoglobin or hemoglobin A has a molecular weight of 64,450 g/mol and contains two pairs of peptide chains (α & β) of which α chain contains 141 and β chain contains 146 amino acids. Fetal hemoglobin (F) is present in very small amounts (Fig 3).

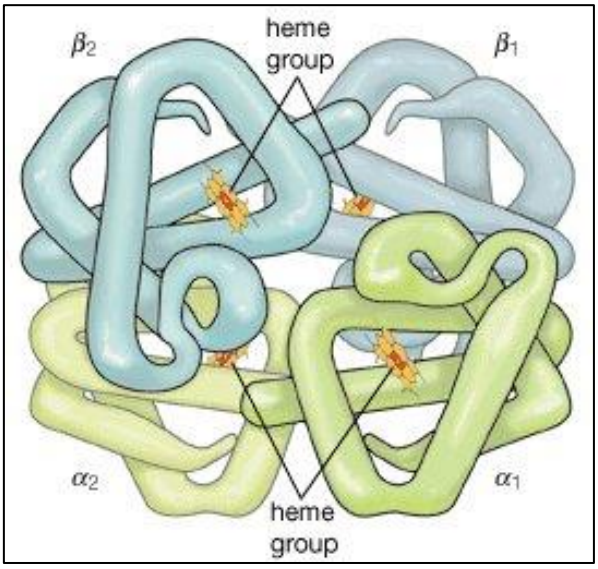

Fig 3: Structure of haemoglobin

Hemoglobin is a protein made up of four polypeptide chains (α_1, α_2, β_1, and β_2). Each chain is attached to a heme group composed of porphyrin (an organic ring like compound) attached to an iron atom. These iron-porphyrin complexes coordinate oxygen molecules reversibly, an ability directly related to the role of haemoglobin in oxygen transport in the blood.

The structural features of the two major types of subunit interfaces in normal adult haemoglobin A ($\alpha_2\beta_2$), i.e., between dimers in the tetramer and between monomers in the dimer and how they change upon O_2 binding, have been elucidated primarily by the studies of Perutz (Perutz 1989), but there have been relatively few reports on the interface properties of the other normal human haemoglobins, including fetal and embryonic haemoglobins.

After the different human haemoglobin monomers are expressed, they join together in various combinations to form a heterodimer. Further assembly to tetramers produces the functional O_2-binding unit of human Hb ($x_2 y_2$) as shown in following equation and in Fig 4.

$2x + 2y \leftrightarrow 2xy \leftrightarrow x_2y_2$ ..... (1)

Where *x* represents α- or ζ-subunits and *y* represents β-, γ-, δ-, or ε-subunits.

Although association of *x* and *y* subunits strongly favours dimer formation, competing self-assembly reactions involving β- or γ-subunits to form β_4 (HbH) and γ_4 (Hb Bart's) can also occur. In human red cells there are eight possible different haemoglobin combinations, but some of these are present in greater amounts than others and not all have been isolated. In addition, there is a temporal factor, known as ontogeny, whereby certain haemoglobins are present at different times during development, i.e., at the embryonic, fetal, and adult stages (Fig 4).

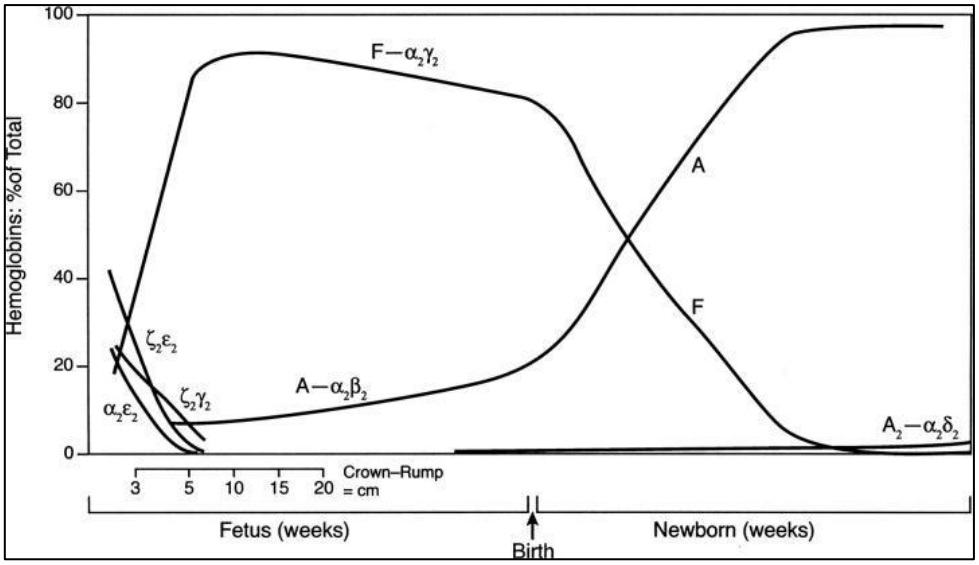

Fig 4: Normal developmental profile of haemoglobins. The names of the normal embryonic haemoglobins are Gower-1 ($\zeta_2\varepsilon_2$), Gower-2 ($\alpha_2\varepsilon_2$), and Portland-1 ($\zeta_2\gamma_2$). Hb Portland-2 ($\zeta_2\beta_2$) is not usually found

The progression and control of haemoglobin ontogeny have been the subjects of intense study for many years, but questions remain as to its mechanism. The haemoglobins present during normal development—$\zeta_2\gamma_2$ (Hb Portland-1), $\zeta_2\varepsilon_2$ (Hb Gower-1), and $\alpha_2\varepsilon_2$ (Hb Gower-2) during the embryonic period; $\alpha_2\gamma_2$ (fetal Hb or HbF) during the fetal stage; and $\alpha_2\beta_2$ (adult Hb or HbA) as well as small amounts of $\alpha_2\delta_2$ (HbA_2) in adults —have some different properties that confer specific advantages during the various stages of development. For example, even though these haemoglobins have very similar overall structural architectures, they have differences in O_2 affinity and in their interactions with allosteric effectors that arise from amino acid substitutions at strategic positions to control the manner in which the subunits fit together. Knowledge of how these subunit interactions differ for various haemoglobins is important in understanding their physiological properties and is reported here.

The order of expression of the globin subunits is determined by their relative gene positions, i.e., ζ → α (2 copies) on chromosome 16 and ε → γ (2 copies) → δ → β on chromosome 11. Hence, combinations of ζ- with ε- or γ- subunits to form embryonic haemoglobins would occur before the appearance of fetal and adult haemoglobins containing α-subunits. However, it is not clear whether there are any dynamics among these haemoglobins and what governs their longevity at the various stages of development. These subjects are also discussed in this report.

The three common embryonic haemoglobins, Hb Portland-1 ($\zeta_2\gamma_2$), Hb Gower-1 ($\zeta_2\varepsilon_2$), and Hb Gower-2 ($\alpha_2\varepsilon_2$), are normally present during the first few months of life, and these have been studied in some detail. However, Portland-2 ($\zeta_2\beta_2$), another embryonic Hb, is found infrequently and mainly in an extreme type of α-thalassemia ("hydrops fetalis") where ζ-chains substitute for α-chains when synthesis of the latter is severely impaired. Hence, Hb Portland-2 has not been extensively studied. In this work, we have evaluated many of its properties to determine whether it has any characteristics that might limit its occurrence. The availability of adequate amounts of this and the other embryonic human haemoglobins from transgenic mice, rather than relying on obtaining human embryonic red cells, has enabled a comprehensive study of the subunit binding properties of all the embryonic haemoglobins and a comparison with the fetal and adult types.

Of the two types of subunit interfaces in human haemoglobin (between dimers in the tetramer and between monomers in the dimer, as shown in Equation 1), the properties and equilibrium constants for the tetramer–dimer interface of many haemoglobins are directly measurable in the CO- or oxy-liganded states where these contacts are not particularly strong. Therefore, the concentrations at which tetramers begin to dissociate to dimers are within the range of available detectors. Tetramer–dimer equilibrium constants have been found to vary considerably among some mutant human haemoglobins and between adult haemoglobin A ($\alpha_2\beta_2$) and fetal haemoglobin F ($\alpha_2\gamma_2$), which has a greatly increased tetramer strength (stability) (Fig 5). However, the normal human haemoglobins including the embryonic haemoglobins have not been systematically studied as a group and compared; thus, it has been generally assumed that their subunit interfaces have similar properties. We report here that the strengths of these interfaces vary quite significantly in the liganded state so that dimers and tetramers are formed to different extents. In the deoxy state where subunit contacts are more extensive and stronger than in the liganded state, equilibrium constants are not easily measured and are not included here.

Hemoglobin[a]	Subunit in common	Classification
A ($\alpha_2\beta_2$)	β	Adult
Portland-2 ($\zeta_2\beta_2$)	β	Embryonic
F ($\alpha_2\gamma_2$)[b]	γ	Fetal
Portland-1 ($\zeta_2\gamma_2$)	γ	Embryonic
Gower-2 ($\alpha_2\varepsilon_2$)	ε	Embryonic
Gower-1 ($\zeta_2\varepsilon_2$)	ε	Embryonic
A_2 ($\alpha_2\delta_2$)[c]		Adult

Fig 5: Types of haemoglobin

Abnormal Haemoglobins

Conditions in which haemoglobin has an abnormal structure include:

- Sickle cell anaemia: Sickle cell anaemia is an inherited condition in which the abnormal haemoglobin results in red blood cells which are shaped like sickles. These red blood cells can get "stuck" in blood vessels resulting in a number of problems.

- Thalassemia: Thalassemia are hereditary abnormal haemoglobins. Both alpha thalassemia and beta thalassemia have a number of different types, and symptoms may vary from none too severe. People with these conditions will often have life-long anaemia, and many require frequent transfusions. Thalassemia intermedia is also termed "non-transfusion dependent thalassemia" and may not be discovered until adulthood.

One amino acid of the normal hemoglobin is replaced by another amino acid, i.e. acidic amino acid is replaced by a basic or a neutral amino acid for the formation of abnormal hemoglobin. The abnormal hemoglobin's are named in alphabetic order as C, D, E, F, G, H, K, L, M, N, O, P, Q, S etc.

A. Hemoglobin C:

This occurs in the blood of some Negroes in West Africa. The abnormality is found in the β chain at position 6, the amino acid glutamic acid is replaced by Lysine. It is characterized by the mild anemia with a tendency to infarction.

B. Hemoglobin S:

This appears among the Negroes of Africa. The abnormality occurs in β chain, glutamic acid at position 6 is replaced by valine. Sickle cell anaemia develops and the RBC becomes long and boat-shaped. The blood becomes more viscous which results in reduced blood flow.

C. Hemoglobin F:

HbF is present in fetus and is replaced by adult hemoglobin as the child grows. It is present only in traces in normal adults, it gets hemolysis rapidly producing a severe anemia called "Thalassemia major".

D. Hemoglobin M:

There are two types of HbM-HbM (Boston) and HbM I Wate which are of clinical interest. The abnormality is found in the α chain, the histidine residues in 58 and 87 position are replaced by tyrosine. Abnormal amounts of met-haemoglobin are found in the blood of persons affected by this condition. This met-haemoglobin is not reduced to hemoglobin by reducing agents.

E. Hemoglobin D:

This occurs rarely. It exists in two forms – Dα and Dβ. The persons having HbD do not show any clinical signs and symptoms.

Probable questions:

1. Write short note on haemoglobin.
2. Differentiate between haemoglobin and myoglobin.
3. Write down the properties of haemoglobin.
4. Describe the structure of haemoglobin,
5. Describe the heme structure.
6. Describe the structure of globin protein.
7. Discuss the ontogeny of haemoglobin.
8. What are the functions of haemoglobin?
9. Discuss the heterogeny of haemoglobin.
10. Discuss about different types of abnormal haemoglobins.
11. What do you mean by carboxyhaemoglobin?
12. What do you mean by carbamino haemoglobin?
13. Write short notes on fetal haemoglobin.

Suggested readings:

1. Review Mechanisms of cooperativity and allosteric regulation in proteins. *Perutz MF Q Rev Biophys. 1989 May; 22(2):139-237.* [PubMed] [Ref list]

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3. Fox, S.I. (2011). Human Physiology. 12th Edn. Mc Graw Hill.

4. Gunstream, S.E. (2010). Anatomy and Physiology with integrated study guide. 4th Edn., Mc Graw Hill

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6. Hill, Wyese and Anderson (2012). Animal Physiology. 3rd Edn. Sineuer Associaes.

7. Randall, Burggren and French Eckert Animal Physiology: Mechanisms and adaptations

Unit VII

LDH: structure, function, ontogeny, heterogeny, control of isozyme patterns

Objective: In this unit you will know about the structure of LDH, its function, ontogeny, heterogeny and control of isozyme patterns

Introduction

Lactate dehydrogenase (LDH) is an enzyme found in most living organisms responsible for the conversion of pyruvate, the end product of glycolysis, into lactic acid. With this conversion, the molecule also uses a unit of the energy transferring molecule NADH, releasing the hydrogen to produce NAD^+, allowing glycolysis to continue.

This conversion is necessary when a cell has little to no oxygen because NAD^+ is necessary to continue making ATP through glycolysis. The enzyme creates lactic acid as an end product, in a fermentation reaction. Lactic acid creates the feeling of your muscles "burning" when you exercise hard because it is building up in the cells. However, the true product of lactate dehydrogenase is more electron carriers, specifically NAD^+.

Lactate dehydrogenase is present within all the cells of your body and works to maintain homeostasis in the absence of oxygen. When a person exercises hard, oxygen levels within muscle tissues drop quickly. In order for the muscle cells to keep functioning, they need to continue creating ATP. Oxygen is typically the final electron receptor at the end of the electron transport chain. Without it, the chain is halted along with ATP synthase.

To continue functioning, the muscles must use the ATP created by the process of glycolysis. This process, to continue, needs electron carriers. Lactate dehydrogenase, in forming lactic acid, removes electrons from NADH to complete the process. In doing so, NAD^+ is created and can then be used in glycolysis to create more ATP. While the process produces far less ATP than the electron transport chain, it allows the cell to continue functioning without ample oxygen.

Isoenzymes: The enzymes that occur in a number of different forms and differ from each other chemically, immunologically and electrophoretically are called "Isoenzymes" or "isozymes". Examples: Isozymes of numerous dehydrogenases, and several oxidases, transaminases, phosphatases, transphosphorylases, proteolytic enzymes, aldolases.

Structure of Lactate Dehydrogenase

Lactate dehydrogenase consists of 4 different subunits (Fig 1), which work as a cohesive unit. These 4 subunits can come in different forms and are coded by different genes. In the human body, there are 5 different isoforms, or versions, of lactate dehydrogenase. These different versions are found in different body tissues, which can help doctors identify where the lactate dehydrogenase come from.

For instance, LDH-1 (lactate dehydrogenase-1) is found in the heart, blood cells, and brain cells. LDH-3 is only found in the lungs. Doctors can recognize the different versions of lactate dehydrogenase by the different subunits they consist of LDH-5 can be seen. The different colours represent the individual protein units which made up the enzyme. Remember that these proteins function as a single unit in the enzyme.

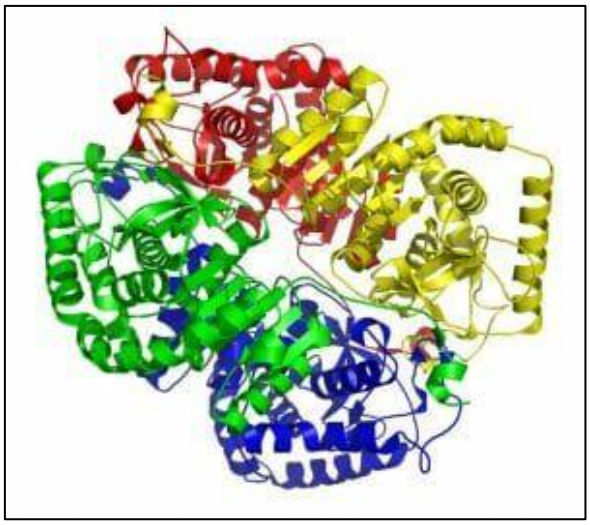

Fig 1: Structure of LDH

The LDH enzyme is present in all cells, but it is concentrated in muscle, liver, and kidney. LDH exists as five isozymes, LDH-1 through LDH-5, each composed of four subunits. Differential LDH isozyme levels are used diagnostically (Markert et al., 1975). Isozyme LDH-1 (4H) has four heart subunits and is the major isozyme in heart. Isozyme LDH-2 (3H1M) has three heart and one muscle subunit and is the major isozyme in the macrophage-monocyte system and serum. The LDH-3 (2H2M) isozyme has two heart and two muscle subunits and is the major isozyme in lungs. Isozyme LDH-4 (1H3M) has one heart and three muscle subunits and is the primary isozyme in kidneys. The LDH-5 (4M) isozyme has four muscle subunits and is the major isozyme in liver and skeletal muscle. The concentration of LDH is elevated in serum as a result of organ infarction and significant cell death that results in loss of cytoplasm. For example, elevations of LDH result from conditions such as hepatitis, shock, hypoxia, extreme hypothermia, and meningitis, among others. The LDH enzyme has often been used in laboratory animals, along with troponin levels, to detect myocardial damage. However, LDH stability is highly susceptible to freezing, and values are affected by storage conditions

Characteristics of Isozymes

1. They catalyse the same reaction but they can be distinguished by physical methods such as electrophoresis or by immunological methods.

2. The difference between some isozymes are due to differences in the quaternary structure of the enzymes, e.g., lactate dehydrogenase exists in five isozymic forms.

3. The isozymic forms of lactate dehydrogenase are tetramers, each is made up from two types of units H and M. The molecular weight of active lactate dehydrogenase is 1,30,000. Only the tetrameric molecule possesses catalytic activity.

4. Splitting and reconstitution of lactate dehydrogenase –I_1 or lactate dehydrogenase- 15 produces on new isozymes. Therefore, each consists of a single subunit. But when a mixture of purified lactate dehydrogenase – I_1 and lactate dehydrogenase – I_5 is subjected to splitting and re- constitution, lactate dehydrogenase – I_2, – I_3 and – I_4 are also produced.

5. Lactate dehydrogenase (LDH) catalyses the transfer of two electrons and one hydrogen ion from lactate to NAD (Fig 2):

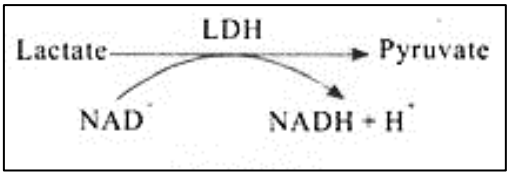

Fig 2: Reaction of LDH

Lactate dehydrogenase catalysis the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. It converts pyruvate, the final product of glycolysis, to lactate when oxygen is absent or in short supply, and it performs the reverse reaction during the Cori cycle in the liver. At high concentrations of lactase, the enzyme exhibits feedback inhibition, and the rate of conversion of pyruvate to lactate is decreased. It also catalyses the dehydrogenation of 2-Hydroxybutyrate, but it is a much poorer substrate than lactate.

LDH in humans uses His (193) as the proton acceptor, and works in unison with the coenzyme (Arg99 and Asn138), and substrate (Arg106; Arg169; Thr248) binding residues. His (193) active site, is not only found in the human form of LDH, but is found in many different animals, showing the convergent evolution of LDH. The two different subunits of LDH: LDHA also known as the M subunit of LDH, and LDHB also known as the H subunit of LDH both retain the same active site, and the same amino acids participating in the reaction. The noticeable difference between the two subunits that make up LDH's tertiary structure is the replacement of alanine (in the M chain) with a glutamine (in the H chain). This tiny but notable change is believed to be the reason the H subunit can bind faster, and the M subunit's catalytic activity isn't reduced when subjected to the same conditions as the H subunit; while the H subunits activity is reduced fivefold.

6. Medical discovery in 1957 had shown that the relative proportions of several lactate dehydrogenase isozymes of human serum were changed significantly in some pathologic conditions.

Lactate Dehydrogenase Function

a. *Producing Energy without Oxygen*

While the process of *oxidative phosphorylation* in the mitochondria produces the most energy, some energy is produced by the breakdown of glucose into pyruvate. This process, glycolysis, requires NAD^+, but produces ATP. The cell can use this small amount of ATP to keep the cell operating until oxygen returns.

Instead of using the pyruvate in the *Krebs cycle*, the pyruvate is converted to lactic acid via lactate dehydrogenase. This process regenerates NAD^+, which is needed to continue glycolysis. Continuing glycolysis produces a small amount of ATP, which allows the cell to survive.

b. *Converting Lactic Acid into Energy*

When oxygen returns, the enzyme can reverse its enzymatic function. This direction creates pyruvate, which can be broken down with oxygen in the mitochondria to produce an abundance of ATP.

While this may seem like a rare event, even simple exercise can lead to oxygen deprivation in certain tissues. Lactate dehydrogenase allows these tissues to continue to produce energy, without oxygen. This causes lactic acid build up in your muscles and tissues, and it is partially responsible for the "burn" felt while you exercise strenuously. When oxygen returns to the muscles, the lactic acid will be converted back into pyruvate and the sensation will cease.

LDH levels across different age groups

Typically, new-borns have the highest levels of the enzyme in their blood, with up to 450 units per liter (U/L). Infants have slightly less, maxing out at somewhere around 250 U/L. Children, who are actively growing, typically have the least, with a maximum of 170 U/L. Adults will tend to have close to 200 U/L. When levels go above this, it is an indication of tissue damage. In the spinal fluid, the typical level is much lower, between 40 and 70 U/L. Elevated levels in the spinal fluid can indicate bacterial infections of the spinal cavity and brain. The treatment of certain cancers is measured in part by the amount and type of lactate dehydrogenase in the system. This can indicate if chemotherapy and radiation are targeting the right tissue types.

Besides cancer and heart failure, this test can be used to identify hypothyroidism, anaemia, pre-eclampsia, meningitis, encephalitis, HIV, and liver or lung disease. The many isoforms of lactate dehydrogenase make it an excellent tool for identifying which tissues are being damaged by a particular disease. This can lead to a more confident diagnosis.

Heterogeny of LDH:

There are five different forms of LDH that are called isoenzymes. They are distinguished by slight differences in their structure. The isoenzymes of LDH are LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5.

Different LDH isoenzymes are found in different body tissues. The areas of highest concentration for each type of isoenzyme are:

- LDH-1: heart and red blood cells
- LDH-2: heart and red blood cells
- LDH-3: lymph tissue, lungs, platelets, pancreas
- LDH-4: liver and skeletal muscle
- LDH-5: liver and skeletal muscle

Usually LDH-2 is the predominant form in the serum. A LDH-1 level higher than the LDH-2 level (a "flipped pattern") suggests myocardial infarction (damage to heart tissues releases heart LDH, which is rich in LDH-1, into the bloodstream). The use of this phenomenon to diagnose infarction has been largely superseded by the use of Troponin I or T measurement.

There are two more mammalian LDH subunits that can be included in LDH tetramers: LDHC and LDHBx. LDHC is a testes-specific LDH protein, which is encoded by the LDHC gene. LDHBx is a peroxisome-specific LDH protein. LDHBx is the read through-form of LDHB. LDHBx is generated by translation of the LDHB mRNA, but the stop codon is interpreted as an amino acid-encoding codon. In consequence, translation continues to the next stop codon. This leads to the addition of seven amino acid acids to the normal LDH-H protein. The extension contains a peroxisomal targeting signal, so that LDHBx is imported into the peroxisome

Ontogeny of LDH

Lactate dehydrogenase (LDH) is a tetrameric enzyme, belonging to the 2-hydroxy acid oxidoreductase family, which increases the rate of the simultaneous inter-conversion of pyruvate to lactate and nicotinamide adenine dinucleotide (NAD)H to NAD+ by 14 orders of magnitude (Fig 3).

The reaction involves the transfer of a hydride ion from NADH to the C2 carbon of pyruvate and is commonly used by cells for anaerobic respiration.

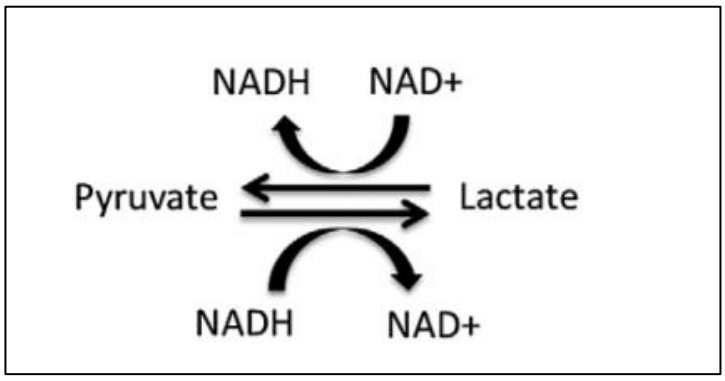

Fig 3: The reaction catalysed by lactate dehydrogenase (LDH). LDH catalyses the reversible conversion of pyruvate and NADH to lactate and NAD+.

There are four LDH genes: LDHA, LDHB, LDHC and LDHD (Fig 4). LDHA, LDHB and LDHC are L isomers, whereas LDHD is a D isomer. The L isomers use or produce L-lactate, which is the major enantiomer found in vertebrates. The human LDHA gene is located on chromosome 11p15.4, the transcribed protein has 332 amino acids, a predicted molecular weight of 37 kDa and 24 splice variants; the human genome also contains several non-transcribed LDHA pseudogenes. Evolutionarily, LDHA and LDHB are thought to have arisen from the duplication of a single LDHA-like LDH gene. LDHC, a testes-specific gene, is also thought to have evolved in mammals from the duplication of the LDHA gene after the A-B duplication. LDHA is also known as the M subunit as it is predominantly found in skeletal muscle, and LDHB is also known as the H subunit as it is predominantly found in the heart. Unlike the other LDH genes, which can form only homotetramers, LDHA and LDHB can form homo- or heterotetramers. There are five isoenzymes of LDH that can be made from the M and H subunits: LDH-1 (4H), LDH-2 (3H, 1M), LDH-3 (2H, 2M), LDH-4 (1H, 3M), and LDH-5 (5M) (Fig 4).

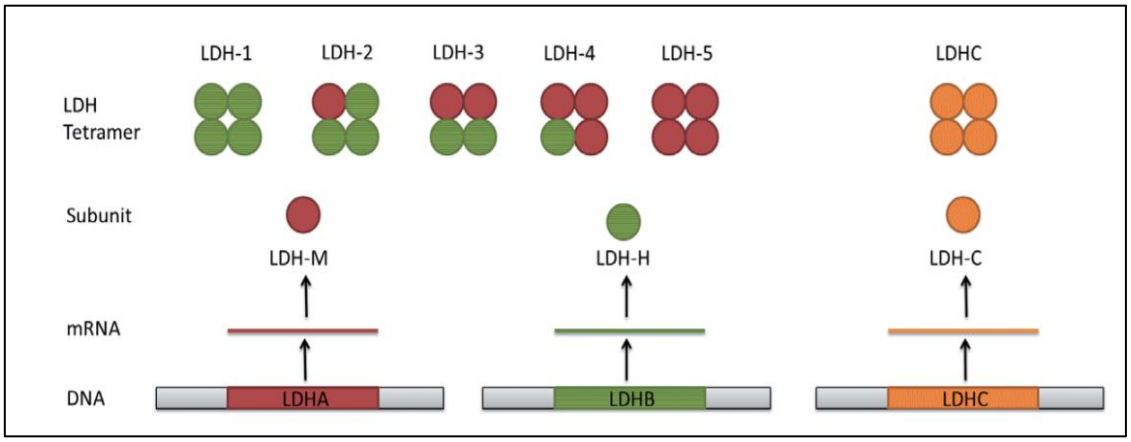

Fig 4: Lactate dehydrogenase (LDH) homo- and tetramer formation. The LDH isoenzymes LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5 are made up of different ratios of LDH-M and LDH-H subunits, transcribed from LDHA and LDHB, respectively. The LDHC tetramer is only made up of LDHC subunits.

LDH-1 and LDH-5 have identical active site regions and only differ in 81 out of 332 amino acid positions, most of which are found in the first 22 and last 38 residues and have a minimal effect on the overall structure. The N-terminus of LDHA is important for structural stability as deletion of up to 10

amino acids from the N-terminus increases instability, flexibility, inactivity and sensitivity to denaturing environments. Although structurally they are very similar, each LDH isoenzyme has different kinetic properties and studies suggest that their distinct kinetics are a result of the differences in charged surface residues bordering the active site. Each LDHA subunit has a net charge of −6 and a higher affinity for pyruvate, preferentially converting pyruvate to lactate and NADH to NAD+, whereas each LDHB subunit has a net charge of +1 and a higher affinity for lactate, preferentially converting lactate to pyruvate and NAD+ to NADH.

Mutation in LDH gene

Mutations of the M subunit have been linked to the rare disease *exertional myoglobinuria*, and mutations of the H subunit have been described but do not appear to lead to disease. In rare cases, a mutation in the genes controlling the production of lactate dehydrogenase will lead to a medical condition known as lactate dehydrogenase deficiency. Depending on which gene carries the mutation, one of two types will occur: either lactate dehydrogenase-A deficiency (also known as glycogen storage disease XI) or lactate dehydrogenase-B deficiency. Both of these conditions affect how the body breaks down sugars, primarily in certain muscle cells. Lactate dehydrogenase-A deficiency is caused by a mutation to the LDHA gene, while lactate dehydrogenase-B deficiency is caused by a mutation to the LDHB gene. This condition is inherited in an autosomal recessive pattern, meaning that both parents must contribute a mutated gene in order for this condition to be expressed. A complete lactate dehydrogenase enzyme consists of four protein subunits. Since the two most common subunits found in lactate dehydrogenase are encoded by the LDHA and LDHB genes, either variation of this disease causes abnormalities in many of the lactate dehydrogenase enzymes found in the body. In the case of lactate dehydrogenase-A deficiency, mutations to the LDHA gene results in the production of an abnormal lactate dehydrogenase-A subunit that cannot bind to the other subunits to form the complete enzyme. This lack of a functional subunit reduces the amount of enzyme formed, leading to an overall decrease in activity. During the anaerobic phase of glycolysis (the Cori Cycle), the mutated enzyme is unable to convert pyruvate into lactate to produce the extra energy the cells need. Since this subunit has the highest concentration in the LDH enzymes found in the skeletal muscles (which are the primary muscles responsible for movement), high-intensity physical activity will lead to an insufficient amount of energy being produced during this anaerobic phase. This in turn will cause the muscle tissue to weaken and eventually break down, a condition known as rhabdomyolysis. The process of rhabdomyolysis also releases myoglobin into the blood, which will eventually end up in the urine and cause it to become red or brown: another condition known as myoglobinuria. Some other common symptoms are exercise intolerance, which consists of fatigue, muscle pain, and cramps during exercise, and skin rashes. In severe cases, myoglobinuria can damage the kidneys and lead to life-threatening kidney failure. In order to obtain a definitive diagnosis, a muscle biopsy may be performed to confirm low or absent LDH activity. There is currently no specific treatment for this condition.

In the case of lactate dehydrogenase-B deficiency, mutations to the LDHB gene results in the production of an abnormal lactate dehydrogenase-B subunit that cannot bind to the other subunits to form the complete enzyme. As with lactate dehydrogenase-A deficiency, this mutation reduces the overall effectiveness in the enzyme. However, there are some major differences between these two cases. The first is the location where the condition manifests itself. With lactate dehydrogenase-B deficiency, the highest concentration of B subunits can be found within cardiac muscle, or the heart. Within the heart, lactate dehydrogenase plays the role of converting lactate back into pyruvate so that the pyruvate can be used again to create more energy. With the mutated enzyme, the overall rate of this conversion is decreased. However, unlike lactate dehydrogenase-A deficiency, this mutation does

not appear to cause any symptoms or health problems linked to this condition. At the present moment, it is unclear why this is the case. Affected individuals are usually discovered only when routine blood tests indicate low LDH levels present within the blood.

LDH Enzyme regulation

This protein may use the morpheein model of allosteric regulation.

Ethanol-induced hypoglycemia

Ethanol is dehydrogenated to acetaldehyde by alcohol dehydrogenase, and further into acetic acid by acetaldehyde dehydrogenase. During this reaction 2 NADH are produced. If large amounts of ethanol are present, then large amounts of NADH are produced, leading to a depletion of NAD+. Thus, the conversion of pyruvate to lactate is increased due to the associated regeneration of NAD+. Therefore, anion-gap metabolic acidosis (lactic acidosis) may ensue in ethanol poisoning. The increased NADH/NAD+ ratio also can cause hypoglycemia in an (otherwise) fasting individual who has been drinking and is dependent on gluconeogenesis to maintain blood glucose levels. Alanine and lactate are major gluconeogenic precursors that enter gluconeogenesis as pyruvate. The high NADH/NAD+ ratio shifts the lactate dehydrogenase equilibrium to lactate, so that less pyruvate can be formed and, therefore, gluconeogenesis is impaired.

Substrate regulation

LDH is also regulated by the relative concentrations of its substrates. LDH becomes more active under periods of extreme muscular output due to an increase in substrates for the LDH reaction. When skeletal muscles are pushed to produce high levels of power, the demand for ATP in regards to aerobic ATP supply leads to an accumulation of free ADP, AMP, and Pi. The subsequent glycolytic flux, specifically production of and pyruvate, exceeds the capacity for pyruvate dehydrogenase and other shuttle enzymes to metabolize pyruvate. The flux through LDH increases in response to increased levels of pyruvate and NADH to metabolize pyruvate into lactate.

Transcriptional regulation

LDH undergoes transcriptional regulation by PGC-1α. PGC-1α regulates LDH by decreasing LDH A mRNA transcription and the enzymatic activity of pyruvate to lactate conversion

Probable questions:

1. Describe the structure of LDH.
2. What is isozymes? Describe its characters.
3. Give a detail discussion about Lactate Dehydrogenase function.
4. Discuss the heterogeny of LDH.
5. Discuss the ontogeny of LDH.
6. Discuss the mutation in LDH gene.

Suggested readings:

1. Sherwood, L. (2013). Human Physiology from cells to systems. 8th Edn., Brooks & Cole

2. Costanzo, L.S. BRS Phyiology.4th Edn. Lippincoat Williams and Wilkins.

3. Fox, S.I. (2011). Human Physiology. 12th Edn. Mc Graw Hill.

4. Gunstream, S.E. (2010). Anatomy and Physiology with integrated study guide. 4th Edn., Mc Graw Hill

Unit VIII

Statistics in biology: Test of hypothesis: Chi-square test, Paired ‘t’ – test

Objective: In this unit you will know about Statistics in biology: Test of hypothesis: Chi-square test, Paired ‘t’ – test

Introduction

Study of biology focuses on living organisms, statistical analyses provide crucial insight into many biological processes. Basic statistical concepts help biologists correctly prepare experiments, verify conclusions and properly interpret results. Many biology courses of study require a course in biostatistics that covers such concepts as randomized trials, hypothesis testing and the use of statistical software.

Establishing Sample Size

An important part of any biological experiment involves correctly choosing samples and selecting the right number of trials. A basic introduction to statistics provides background in statistical randomness and the law of large numbers. When conducting a study of whether insects prefer to eat American elm leaves or Princeton elm leaves, for example, using a properly randomized sample of both types of leaves helps control for confounding factors. For example, choosing a small number of leaves from the American elm, if those were all from a single tree, might result in a tree that was unusually filled with insects and would bias the results. Selecting many hundreds of leaves from a random sample of trees, however, reduces this type of error.

Hypothesis Testing

When conducting experiments with a large sample, a biologist must make sure that a conclusion is statistically significant. One such experiment might involve examining whether smoking leads to cancer. By examining the means of two groups -- one that smokes and one that doesn't -- a biologist might find that the smokers more commonly had cancer. Means, however, reflect the center of a distribution of data, and hypothesis testing involves examining the spread of that distribution. If the data is heavily clustered around the mean, the mean number is a reliable indicator; if the data is heavily spread out, the mean is less reflective of overall trends and should be considered in that context.

Interpreting Data Analyses

After finishing an experiment or observation, biologists need statistics to draw proper conclusions. For example, comparing the data from two groups of plants -- one that was watered and one that was not -- can lead to erroneous conclusions. A biologist, for example, might simply record the mean height of these two sets of plants and conclude that the watered plants grew taller. This, however, does not account for other statistical measures, such as variance. The nonwatered plants might have grown less tall on average, but perhaps their heights varied more widely than their watered counterparts, which is important data to relay in a conclusion.

Statistical Software

Very large data sets cannot easily be processed by hand. In many biological situations, such as those in ecology that use large sample sizes, using statistical software makes data processing more expedient. Data programs include Stata; Statistical Analysis System, or SAS; and Statistical Product

and Service Solution, or SPSS. Most introductory statistics classes will use these software products, which can involve learning programming languages.

How to determine the appropriate statistical test

I find that a systematic, step-by-step approach is the best way to decide how to analyse biological data. I recommend that you follow these steps:

1. Specify the biological question you are asking.
2. Put the question in the form of a biological null hypothesis and alternate hypothesis.
3. Put the question in the form of a statistical null hypothesis and alternate hypothesis.
4. Determine which variables are relevant to the question.
5. Determine what kind of variable each one is.
6. Design an experiment that controls or randomizes the confounding variables.
7. Based on the number of variables, the kinds of variables, the expected fit to the parametric assumptions, and the hypothesis to be tested, choose the best statistical test to use.
8. If possible, do a power analysis to determine a good sample size for the experiment.
9. Do the experiment.
10. Examine the data to see if it meets the assumptions of the statistical test you chose (primarily normality and homoscedasticity for tests of measurement variables). If it doesn't, choose a more appropriate test.
11. Apply the statistical test you chose, and interpret the results.
12. Communicate your results effectively, usually with a graph or table.

Some important terms in statistics

1. **Statistical hypothesis:** In statistics, hypothesis is an assumption about a population parameter.
2. **Test of hypothesis:** The procedure which enables us to decide whether a certain hypothesis is true or not. Statistically it is two types i.e. null hypothesis and alternative hypothesis.
3. **Null hypothesis (H_0):**
   A statistical hypothesis which is set up (assumed) and whose validity is tested for possible rejection on the basis of sample observation is called a 'null hypothesis'
   - ✓ It is denoted byH_0 and tested against alternatives.
   - ✓ Test of hypothesis deal with rejection or acceptance of null hypothesis only
   - ✓ Prof R.A. Fisher remarked '*null hypothesis' is the hypothesis which is to be tested for possible rejection under the assumption it is true'*
4. **Alternative hypothesis (H_1):**
   The negation of null hypothesis is called the alternative hypothesis.
   - ✓ It is denoted by H_1 and H_∞
   - ✓ It is not tested, but its acceptance (rejection) depends on the rejection (acceptance) of the null hypothesis.
   - ✓ Alternate hypothesis contradict the null hypothesis

5. **Level of significance (α):** Maximum probability of rejecting a true null hypothesis is called level of significance. It is denoted by α.

   In practice, generally we choose 5% (0.05 or $p<0.05$) and 1% (0.01 or $p<0.01$) level of significance, although 2%, ½ % levels are also taken sometimes. The 5% and 1% levels of significance also means we are 95% & 99% confident about our decision taken before testing of hypothesis, or probability of making type-I error is 5% and 1% respectively

6. **Type-I error or error of first kind:** It is an incorrect rejection of true null hypothesis.

7. **Type-II error or error of second kind:** It is an incorrect acceptance of false null hypothesis.

Chi-square (χ^2) test

You use the chi-square test of goodness-of-fit when you have one nominal variable, you want to see whether the number of observations in each category fits a theoretical expectation, and the sample size is large.

When to use it Use the chi-square test of goodness-of-fit when you have one nominal variable with two or more values (such as red, pink and white flowers). You compare the observed counts of observations in each category with the expected counts, which you calculate using some kind of theoretical expectation (such as a 1:1 sex ratio or a 1:2:1 ratio in a genetic cross). If the expected number of observations in any category is too small, the chi-square test may give inaccurate results, and you should use an exact test instead.

Definition: It is a statistical method mainly used to test the goodness of fit or independence of attributes between variables. It is denoted by χ^2.

Degree of freedom: Degree of freedom is the number of values / observations in a study that are free to vary. It is the number of independent observations in a set.

Null hypothesis: The statistical null hypothesis is that the number of observations in each category is equal to that predicted by a biological theory, and the alternative hypothesis is that the observed numbers are different from the expected.

Example-1. Consider, only nine courses are offered for a student to be a graduate. There are 8 d.f. as because he can choose any one out of eight courses, but the 9^{th} one is obligatory for him.

Calculation of d.f.: Degree of freedom is equal to the number of values of a data set minus one. i.e., d.f.= n-1, where n = number of values in a data set. The d.f. for sample size n = 9 will be 8.
If there are two samples of sizes n_1 and n_2 with specified means x_1 & x_2 , then the d.f. would be $(n_1 + n_2 -2)$. For contingency table with h rows and k columns, the d.f is (h-1) (k-1).

Example: A 2 X 2 contingency table is as below:

Literacy	Literate	Illiterate	Total
HTN	20	25	45
Normotensive	80	130	210
Total	100	155	T=255

Here, d.f.= (2-1) x (2-1) = 1

Uses of chi-square test: Chi-square test can be used in both large sample (>30) & small sample tests (<30). It is used to-

1. Test for goodness of fit (or Pearsonian χ^2).

2. Test for independence of attributes.

3. Test for a specified S.D. (small sample test).

Test for goodness of fit: Chi-square for test of goodness of fit can be done by using following formula-

$$\chi^2 = \frac{\sum(O-E)^2}{E}$$

Where, O = Observed frequency and E= Expected frequency.

Conclusion/Decision: If the observed value exceeds tabulated value of χ^2 at a given level of significance (5% or 1%), the null hypothesis (H_0) is rejected. Otherwise accepted.

Problem-I: In the experiment on pea-breeding, Mendel obtained the following frequency of seeds: Round & yellow-315;
Wrinkled & yellow – 101;
Round & green-108; and
Wrinkled & green-32; Total-556. Theory predicts that the frequency should be in the proportion of 9:3:3:1. Examine the correspondence between theory and observations. (Given that value of χ^2 for d.f. =3 is 7.815).

Solution: H_0: It is assumed that data agree with the theory. Expected frequencies are calculated below-

Class	Observed frequency (O)	Expected frequency (E)	$(O-E)^2 / E$
Round & yellow	315	9 X 556 /16 = 313	4/313 = 0.01
Wrinkled &	101	3x556 / 16 = 104	9/104 = 0.09
Round & green	108	3x556 / 16 = 104	16/104 = 0.15
Wrinkled & green	32	1 x 556 / 16 = 35	9/35 = 0.26
Total	556	556	$\sum (O-E)^2/E=0.51$

Here, total no. of classes is 4.
So, d.f. = 4-1 = 3.
& we know that
$\chi^2 = \sum (O-E)^2 / E$
= 0.01 + 0.09 + 0.15 + 0.26 = 0.51

Conclusion: The calculated value of chi-square (0.51) is less than that of given value of chi-square (7.815) for d.f, 3 at the 5% level of significance. Therefore, H_0 is accepted which means the data are in agreement with the hypothesis, i.e., the observation support the theory.

Problem-II : Nephropathy was observed in 100 cases of each class of diabetes, divided into 4 classes as per severity of disease.

Class	I	II	III	IV	Total
f_0	8	15	14	7	320

Is this inequality in different classes due to severity? (Given $\chi^2_{0.05\,,\,\text{d.f. }3} = 7.81$).

Solution: Assuming that the H_0 is true, i.e., inequality is not due to severity of diabetes. The probability of expected frequencies of nephropathy in each class of diabetes should be the same i.e., 8+15+14+7 / 4 = 44/4 =11.

Class	Observed frequency (O)	Expected frequency (E)	$(O-E)^2 / E$
I	8	11	9/11=0.82
II	15	11	16/11 = 1.45
III	14	11	9/11 =0.82
IV	7	11	16/11 =1.45
Total	44	44	$\sum (O-E)^2/E$= 4.54

Here, total no. of classes are 4. So, d.f. = 4-1 = 3. And

$\chi^2 = \sum (O-E)^2 / E = 4.54$

Conclusion: The calculated value of chi-square (4.54) is less than that of given value of chi-square (7.81) for d.f, 3 at the 5% level of significance.

Therefore, H_0 is accepted which means the data are in agreement with the hypothesis, i.e., the severity of the disease in diabetics is not due to severity of the disease.

Problem-III : A die was thrown 60 times with the following results:

Face	1	2	3	4	5	6	Total
f_o	6	10	8	13	11	12	60

Are the data consistent with the hypothesis that the die is unbiased? (Given $\chi^2_{0.01, 5} = 15.09$).

Solution:

H_0: It is assumed that the die is unbiased.
H_1: The die is biased.
The probability of expected frequencies should be the same in unbiased coin, i.e., 60/6 = 10.

Class	Observed frequency (O)	Expected frequency (E)	$(O-E)^2 / E$
1	6	10	16/10=1.6
2	10	10	0/10 = 0.0
3	8	10	4/10 =0.4
4	13	10	9/10 =0.9
5	11	10	1/10 =0.1
6	12	10	4/10 =0.4
Total	556	556	$\sum (O-E)^2/E$= 3.4

Here, total no. of classes are 6. So, d.f. = 6-1 = 5. And
$\chi^2 = \sum (O-E)^2 / E$
= 1.6 + 0.0 + 0.4 + 0.9 +0.1+0.4
= 3.4

Conclusion: The calculated value of chi-square (3.4) is less than that of given value of chi-square (15.09) for d.f, 5 at the1% level of significance.

Therefore, H_0 is accepted which means the data are in agreement with the hypothesis, i.e., the die is unbiased.

Student's t-test

In statistics, a method of testing hypotheses about the mean of a small sample drawn from a normally distributed population when the population standard deviation is unknown.

In 1908 William Sealy Gusset, an Englishman publishing under the pseudonym Student, developed the *t*-test and *t* distribution. The *t* distribution is a family of curves in which the number of degrees of freedom (the number of independent observations in the sample minus one) specifies a particular curve. As the sample size (and thus the degrees of freedom) increases, the *t* distribution approaches the bell shape of the standard normal distribution. In practice, for tests involving the mean of a sample of size greater than 30, the normal distribution is usually applied.

It is usual first to formulate a null hypothesis, which states that there is no effective difference between the observed sample mean and the hypothesized or stated population mean—i.e., that any measured difference is due only to chance. In an agricultural study, for example, the null hypothesis could be that an application of fertilizer has had no effect on crop yield, and an experiment would be performed to test whether it has increased the harvest. In general, a *t*-test may be either two-sided (also termed two-tailed), stating simply that the means are not equivalent, or one-sided, specifying whether the observed mean is larger or smaller than the hypothesized mean. The test statistic *t* is then calculated. If the observed *t*-statistic is more extreme than the critical value determined by the appropriate reference distribution, the null hypothesis is rejected. The appropriate reference distribution for the *t*-statistic is the *t* distribution. The critical value depends on the significance level of the test (the probability of erroneously rejecting the null hypothesis).

Type

1. Unpaired *t* test
2. Paired *t* test

Paired t test

It is applied to paired data of independent observation from one sample only when each individual gives a pair observations.

- To study the role of a factor or cause when the observations are made before and after its play
- To compare the effects of two drugs
- To compare the results of two different laboratory technique
- To compare observations made at two different laboratory technique.

Working procedure

- Find the difference in each set of paired observations before and after ($X_1 - X_2 = D$)
- Calculate the mean of the difference ($\overline{D}$)
- Work out the SD of differences and then SE of mean from the same SD/√N
- Calculate the 't' value by substituting the above values in the formula.

$$t = \frac{\bar{D} - 0}{SD/\sqrt{n}} = \frac{\bar{D}}{\frac{SD}{\sqrt{n}}}$$

$$SD = \sqrt{\frac{\sum D^2 - \frac{(\sum D)^2}{n}}{n-1}}$$

- Determine the degrees of freedom. Being one and the same sample df should be n – 1
- Compare the calculated value with the table value at a particular degrees of freedom

Problem I: Ten students were given intensive coaching in Statistics. The scores obtained in 1st and 5th test are given below:

Sl no.	1	2	3	4	5	6	7	8	9	10
Marks in 1st	50	52	53	60	65	67	48	69	72	80
Marks in 2nd	65	55	65	65	60	67	49	82	74	86

Does the score from test 1st to test 5th show an improvement? Test at 5% level of significance.

Solution:

Null hypothesis: No improvement has been occurred (assume)
Alternative hypothesis: Improvement (assume)

Calculation:

Sl no.	Marks in 1st test (X_1)	Marks in 5th test (X_2)	Difference ($X_1 - X_2 = D$)	D^2
1	50	65	-15	225
2	52	55	-3	09
3	53	65	-12	144
4	60	65	-5	25
5	65	60	5	25
6	67	67	0	0
7	48	49	-1	1
8	69	82	-13	109
9	72	74	-2	04
10	80	86	-6	36
			$\sum D = -52$	$\sum D^2 = 638$

$$\bar{D} = \frac{\sum D}{n} = \frac{-52}{10} = -5.2$$

$$SD = \sqrt{\frac{\sum D^2 - \frac{(\sum D)^2}{n}}{n-1}}$$

$$= \sqrt{\frac{638 - \frac{(-52 \times -52)}{10}}{10-1}}$$

$$= \sqrt{\frac{638 - 270.4}{9}}$$

$$= \sqrt{\frac{367.6}{9}} = \frac{19.17}{3} = 6.391$$

$$SE \text{ of difference} = \frac{S}{\sqrt{n}} = \frac{6.391}{\sqrt{10}} = \frac{6.391}{3.16} = 2.022$$

$$t = \frac{\bar{D}}{SE} = \frac{-5.2}{2.022} = 2.57$$

Level of significance = 0.05
Critical value = The critical value of 't' at 0.05 for 10 – 1= 9 df = i.e., $t_{0.05, 9} = 2.26$

Decision: Since calculated 't' = 2.57 > tabulated value $t_{0.05, 9} = 2.26$. So the null hypothesis is rejected i.e., alternative hypothesis is accepted.

Problem II: Ten rats were fed with rice in first months and body weights of the rats were recorded. In the next months they were fed with grams and their weights were measured again. The respective weights of ten rats in two months are as follows.

Weight in 1st months		50	60	58	52	51	62	58	55	50	65
Weight in 2nd months		56	58	68	61	56	59	64	60	50	62

Test the given data to find the impact of grams in rat's nutrition.

Solution:

Null hypothesis: Grams have no impact in rat's nutrition
Alternative hypothesis: Grams have impact in rat's nutrition

Calculation:

Sl no.	Weight in 1st month (X_1)	Weight in 2nd month (X_2)	Difference ($X_1 - X_2$ = D)	D^2
1	50	56	-6	36
2	60	58	2	04
3	58	68	-10	100
4	52	61	-9	81
5	51	56	-5	25
6	62	59	3	09
7	58	64	-6	36
8	55	60	-5	25
9	50	50	0	0
10	65	62	3	09
			$\sum D = -33$	$\sum D^2 = 325$

$n = 10$

$$\bar{D} = \frac{\Sigma D}{n} = \frac{-33}{10} = -3.3$$

$$S.D. = \sqrt{\frac{\Sigma D^2 - \frac{(\Sigma D)^2}{n}}{n-1}} = \sqrt{\frac{325 - \frac{(-33)^2}{10}}{10-1}} = \sqrt{\frac{325 \quad \frac{1089}{10}}{9}}$$

$$= \sqrt{\frac{325 - 108.9}{9}} = \sqrt{\frac{216.1}{9}} = \sqrt{24.1} = 4.9$$

$$SE = \frac{SD}{\sqrt{n}} = \frac{4.9}{\sqrt{10}} = \frac{4.9}{3.16} = 1.55$$

$$|t| = \frac{3.3}{1.5} = 2.13.$$

Level of significance: 0.05 level
Critical value: Tabulated value of $|t|$ at 0.05 levels at df (10-1) =9 is 2.26

Decision: The calculated value of $|t|$ is $2.13 < t_{0.05, 9} = 2.26$
So the null hypothesis is accepted i.e., grams have no impact in rat nutrition.

Non parametric tests

Nonparametric statistics is the branch of statistics that is not based on parameterized families of probability distributions. The term nonparametric statistics is to indicate that the population could not be specified by a finite number of parameters. A term which is often used interchangeably with non-parametric is **distribution-free** (distributed parameters are not dependent on distribution function of population). Nonparametric statistics includes both descriptive statistics and statistical inference.

Methods:

Non-parametric (or **distribution-free**) **methods:** The most frequently used tests are:

- *Anderson–Darling test*: tests whether a sample is drawn from a given distribution
- *Log rank test*: compares survival distributions of two right-skewed, censored samples
- *Mann–Whitney U or Wilcoxon rank sum test*: tests whether two samples are drawn from the same distribution, as compared to a given alternative hypothesis.
- *Sign test*: tests whether matched pair samples are drawn from distributions with equal medians.
- *Spearman's rank correlation coefficient*: measures statistical dependence between two variables using a monotonic function
- *Wilcoxon signed-rank test*: tests whether matched pair samples are drawn from populations with different mean ranks

Application & purposes of non-parametric tests

- Non-parametric methods are widely used for studying populations that take on a ranked order. The use of non-parametric methods may be necessary when data have a ranking but no clear numerical interpretation, such as when assessing preferences.
- As non-parametric methods make fewer assumptions, their applicability is much wider than the corresponding parametric methods. In particular, they may be applied in situations where less is known about the application in question.
- Another justification for the use of non-parametric methods is simplicity. In certain cases, even when the use of parametric methods is justified, non-parametric methods may be easier to use due to this simplicity and greater robustness.
- Non-parametric tests are rather quick and easy to use because they do not require laborious computations as in parametric tests.

Rank correlation

When two qualities (e.g., intelligence and efficiency of a salesman) of an individual are simultaneously ranked (e.g., 1, 2, 3…) to calculate their product-moment (degree of salesmanship) correlation coefficient (r) more precisely (but not error free) are called *rank correlation*. The individuals are said to be ranked and the numbers allotted to a particular candidate is called *rank*. Therefore, each candidate has a pair of ranks, one in each character.

Spearman's Rank correlation

The correlation coefficient between two series of ranks is known as Rank correlation coefficient. It is calculated by using following **Spearmaman's formula** of rank correlation coefficient-

$$R = 1 - \frac{6\sum d^2}{n^3 - n}$$

Where, d = differe

Problem-1: In a contest two judges ranked seven candidates in order to their preference as in the table below:

Candidates	A	B	C	D	E	F	G
Judge-I	2	1	4	5	3	7	6
Judge-II	3	4	2	5	1	6	7

Calculate the rank correlation coefficient.

Solution:

Candidates	Judge-I (x)	Judge-II (y)	d = x-y	d^2
A	2	3	-1	1
B	1	4	-3	9
C	4	2	2	4
D	5	5	0	0
E	3	1	2	4
F	7	6	1	1
G	6	7	-1	1
TOTAL			$\sum d = 0$	$\sum d^2 = 20$

Calculation:

Here, n = 7 and $\sum d^2 = 20$

$\therefore R = 1 - \frac{6\sum d^2}{n^3 - n}$

$= 1 - \frac{6 .20}{7^3 - 7}$

= 1- 120/336

$\therefore$ R= 0.64

Wilcoxon Signed Rank (Matched-Pair) Test

Introduction

The Wilcoxon signed rank sum test is another example of a non-parametric or distribution free test. As for the sign test, the Wilcoxon signed rank sum test is used to test the null hypothesis that the median of a distribution is equal to some value.

It can be used

a) In place of a one-sample t-test

b) In place of a paired t-test or

c) For ordered categorical data where a numerical scale is inappropriate but where it is possible to rank the observations.

Carrying out the Wilcoxon signed rank sum test

Case 1: Paired data

1. State the null hypothesis - in this case it is that the median difference, M, is equal to 0.

2. Calculate each paired difference, di = xi − yi, where xi, yi are the pairs of observations.

3. Rank the di, ignoring the signs (i.e. assign rank 1 to the smallest |di|, rank 2 to the next etc.)

4. Label each rank with its sign, according to the sign of di.

5. Calculate W+, the sum of the ranks of the positive di, and W−, the sum of the ranks of the negative di. (As a check the total, $W^+ + W^-$, should be equal to n(n+1)/2, where n is the number of pairs of observations in the sample).

Case 2: Single set of observations

1. State the null hypothesis - the median value is equal to some value M.

2. Calculate the difference between each observation and the hypothesised median, di = xi − M.

3. Apply Steps 3-5 as above.

4. Under the null hypothesis, we would expect the distribution of the differences to be approximately symmetric around zero and the distribution of positives and negatives to be distributed at random among the ranks.

5. Under this assumption, it is possible to work out the exact probability of every possible outcome for W. To carry out the test, we therefore proceed as follows:

6. Choose **W = min (W−, W+)**.

7. Use tables of critical values for the Wilcoxon signed rank sum test to find the probability of observing a value of W or more extreme. Most tables give both one-sided and two-sided p-values. If not, double the one-sided p-value to obtain the two-sided p-value. This is an exact test.

Normal approximation

In case sample size exceeds 25 (i.e., n> 25), the sampling distribution of W is taken as approximately normal with **mean μ_w = n(n + 1) / 4** and **S.D. σ_w =** $\sqrt{\{n(n+1)(2n+1)/24\}}$. Where, n = (number of given matched pairs) – (number of dropped out pairs, if any) and in this situation the test statistics Z is worked out as under:

$$\mathbf{Z} = \frac{W-\mu w}{\sigma w}$$

$$= \frac{W-\frac{n(n+1)}{4}}{\sqrt{n(n+1)(2n+1)/24}}$$

Dealing with ties

There are 2 types of tied observations that may arise when using the Wilcoxon signed rank test:
• Observations in the sample may be exactly equal to M (i.e. 0 in the case of paired differences). Ignore such observations and adjust n accordingly.

• Two or more observations/differences may be equal. If so, average the ranks across the **tied observations** and reduce the variance by $(t^3-t) / 48$ for each group of t tied ranks.

Example-1: The following data gives the hind leg and foreleg length of 10 deers:

Case	1	2	3	4	5	6	7	8	9	10	Total
Hind leg length (xi)	142	140	144	144	142	146	149	150	142	148	
Foreleg length (yi)	138	136	147	139	143	141	143	145	136	146	

Test the hypothesis that there is no difference between fore & hind leg of deers at 5% level.

Solution:

Deer	Hind leg length (x_i)	Foreleg length (y_i)	Differences $d_i = x_i - y_i$	Rank of $\|d_i\|$	Signed rank of $\|d_i\|$	W^+	W^-
1	142	138	4	4.5	4.5	4.5	
2	140	136	4	4.5	4.5	4.5	
3	144	147	-3	3	-3		-3
4	144	139	5	7	7	7	
5	142	143	-1	1	-1		-1
6	146	141	5	7	7	7	
7	149	143	6	9.5	9.5	9.5	
8	150	145	5	7	7	7	
9	142	136	6	9.5	9.5	9.5	
10	148	146	2	2	2	2	
Total						$\|\sum W^+\|=51$	$\|\sum W^-\|= 4$

Here, **H_0 :** (hind leg length = foreleg length)
H_1 : (hind leg length = foreleg length)
α = 5% level of significance
W = min (W^+, W^-) = min (51, 4) = 4
The table value of **W** at 5% level is 8 (when n = 10)

Conclusion: The observed value of W i.e., 4 is less than that of tabulated value of W i.e., 8 when n=10 at 5% level of significance. Hence, H_0 = rejected i.e., there is no significant difference between hind and foreleg length.

Probable questions:

1. What is null hypothesis?
2. What is alternate hypothesis?
3. What do you mean by level of significance?
4. What is degree of freedom?
5. Practice mathematics

Suggested readings:

1. Principles and Practice of Biostatistics - E-book28 April 2017 by Antonisamy, B and Prasanna S. Premkumar

2. Research Methodology and Biostatistics: A Comprehensive Guide for Health Care Professionals Kindle Edition by Sharma Suresh

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-IV

ELECTIVE THEORY PAPER

CYTOGEETICS AND MOLECULAR BIOLOGY

ZET-403

SELF LEARNING MATERIAL

DIRECTORATE OF OPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Director
Prof. Manas Mohan Adhikary
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE THEORY PAPER (ZET -403)

CYTOGENETICS AND MOLECULAR BIOLOGY

Unit I -Epigenetics

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 404 (CYTOGENETICS AND MOLECULAR BIOLOGY)	I	**A brief history of epigenetics - overview and concepts; chromatin modifications and their mechanism of action, concept of 'histone-code' hypothesis, epigenetics in *saccharomyces cerevisiae*, position effect variegation, heterochromatin formation, and gene silencing in *Drosophila.***	1.5	1	1	
	II	**Fungal models for epigenetic research: *Schizosaccharomyces pombe* and *Neurospora crassa*; RNAi and heterochromatin assembly, role of noncoding RNAs; epigenetic regulation in plants.**		1	1	
	III	**Chromatin structure and epigenetics marks - transcriptional silencing by polycomb group proteins , transcriptional regulation by trithorax group proteins, histone variants and epigenetics, epigenetic regulation of chromosome inheritance,**		1	1	

	IV	**Epigenetic regulation of the X chromosomes in *C.elegans*, dosage compensation in *Drosophila*, dosage compensation in mammals; types mechanism of chromatin remodeling.**		**1**	**1**	
	V	**Epigenetics and genome imprinting - DNA methylation in mammals, genomic imprinting in mammals,**		**1**	**1**	
	VI	**Nuclear transplantation and the reprogramming of the genome. epigenetics and human disease, epigenetic determinants of cancer.**		**1**	**1**	

Unit-I

A brief history of epigenetics - overview and concepts; chromatin modifications and their mechanism of action, concept of 'histone-code' hypothesis, epigenetics in *saccharomyces cerevisiae*, position effect variegation, heterochromatin formation, and gene silencing in *Drosophila.*

Objective: In this unit we will discuss brief history of epigenetics. We will give emphasis to chromatin modifications and their mechanism of action. Epigenetics in *Saccharomyces cerevisiae*, gene silencing in *Drosophila* will also be discussed in this unit.

Epigenetics: Epigenetics is the study of heritable changes in gene expression (active versus inactive genes) that do not involve changes to the underlying DNA sequence — a change in phenotype without a change in genotype — which in turn affects how cells read the genes. Epigenetic change is a regular and natural occurrence but can also be influenced by several factors including age, the environment/lifestyle, and disease state. Epigenetic modifications can manifest as commonly as the manner in which cells terminally differentiate to end up as skin cells, liver cells, brain cells, etc. Or, epigenetic change can have more damaging effects that can result in diseases like cancer. At least three systems including DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are currently considered to initiate and sustain epigenetic change. New and ongoing research is continuously uncovering the role of epigenetics in a variety of human disorders and fatal diseases.

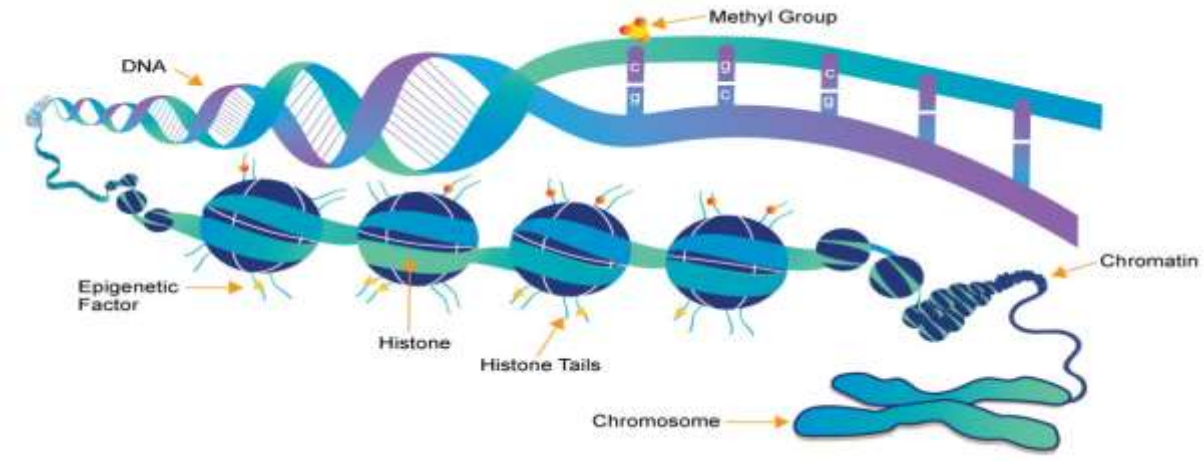


Fig: Representation of the chromatin structure, including histones and DNA, which become available to epigenetic marks.

The Evolving Landscape of Epigenetic Research: A Brief History

What began as broad research focused on combining genetics and developmental biology by well-respected scientists including **Conrad H. Waddington** and Ernst Hadorn during the mid-twentieth century has evolved into the field we currently refer to as epigenetics. The term epigenetics, which was coined by Waddington in 1942, was derived from the Greek word "epigenesis" which originally described the influence of genetic processes on development. During the 1990s there became a renewed interest in genetic assimilation. This led to elucidation of the molecular basis of Conrad Waddington's observations in which environmental stress caused genetic assimilation of certain phenotypic characteristics in *Drosophila* fruit flies. Since then, research efforts have been focused on unraveling the epigenetic mechanisms related to these types of changes.

Currently, DNA methylation is one of the most broadly studied and well-characterized epigenetic modifications dating back to studies done by Griffith and Mahler in 1969 which suggested that DNA methylation may be important in long term memory function.4 Other major modifications include chromatin remodeling, histone modifications, and non-coding RNA mechanisms. The renewed interest in epigenetics has led to new findings about the relationship between epigenetic changes and a host of disorders including various cancers, mental retardation associated disorders, immune disorders, neuropsychiatric disorders and paediatric disorders.

The human genome is composed of billions of sequence arrangements containing a bioinformatics code that controls how genes are expressed. This code is further dependent upon heritable non-static epigenetic arrangement of histone scaffolding that surrounds the DNA and comprises the "epigenome." The historical transitional evolution of the human genome is believed to have occurred through a number of processes, one being the altered sequence and re-arrangement of transposable elements located at segments of non-coding DNA. It is believed that the greater the complexity of an organism, the greater amount of non-coding DNA. In humans, protein-coding regions of DNA account for <1.6% of the genome. Transposable elements, also referred to as "jumping genes," have accumulated throughout millions of years as evolutionary ancient DNA in the form of transposons and retrotransposons, which are reverse transcribed long-terminal repeat (LTR) retroviruses. Today, active non-LTR retrotransposons (i.e., Alu and LINEs) perpetuate transgenerational genetic diversities through genomic DNA variation among humans. While the evolution of DNA occurs at a slow pace, expedient heritable changes to the epigenome allow dynamic and flexible modification to suit rapid environmental adaptation. While the epigenome has more influence on the temporal phenotype, the collective effects of change to the genome and the epigenome contribute to observable physical or biochemical characteristics of an organism.

Throughout the life cycle, dynamic epigenetic control over the phenotype is influenced by a time component responsible for maturation and senescence from conception to adulthood. Environmental epigenetic factors affecting long-term phenotypic change are largely initiated during in utero/perinatal periods, when introduction to the external world is being established. It is believed that since epigenetic patterns are inherited through mitosis, the earlier

the stage of development, the more critical the environmental impact on the resulting phenotype. During fetal development, environmental cues can induce the modification of a pliable epigenome, which can result in long-term changes in gene expression that occur in a self-sustaining manner in the absence of the original stimulus. Adverse gestational conditions that arise from inadequate healthcare, poor nutrition, socioeconomic disadvantage and racial disparities are often associated with long-lasting phenotypic consequences in adults, yielding greater risk of diabetes and heart disease, as well as low birth weight and congenital defects in progeny. It is now becoming evident that these effects are inextricably linked to altered epigenetic patterns. Offspring exposed to gestational malnutrition due to extended famine in certain populations also show higher prevalence of adult onset obesity and schizophrenia, tantamount to altered DNA methylation patterns for specific genes such as *insulin like growth factor 1/2* and the obesity factor gene *leptin*. Altered epigenetic patterns acquired during early development involve changes in DNA methylation patterns, genomic imprinting, histone modifications and the establishment of specific expression profiles of non-coding miRNAs.

Given the enormous impact of early epigenetic programming and the serious nature of related developmental conditions, such as Prader-Willi, Angelman and Beckwith-Wiedemann syndromes, neural tube defects, adult onset psychiatric disorders, obesity, cancer and schizophrenia, considerable attention is given to the "nurture of the epigenome" prior to birth. A number of community outreach projects such as the CDC's National Centre on Birth Defects and Developmental Disabilities promote awareness about reducing the risk of epigenetic related defects such as spina bifida by suggesting an adequate intake of epigenetic-related nutrients (e.g., choline, vitamin B_{12}, B_6 and folate) during pregnancy. The epigenome appears to remain pliable after birth and during the first years of life. This is evidenced by correlations described in infants exposed to stress or lack of emotional nurturing who show overactive hypothalamic-pituitary-adrenal stress response, glucocorticoid feedback or decreased hypothalamic corticotropin-releasing factor. Once established in the offspring, epigenetic marks can become transgenerational, continuing transmittance to future descendants—including the very trait of maternal nurturing in females. The longevity of transgenerational epigenomic inheritance pattern is further influenced by the severity and repetition of a similar environmental stimulus among individuals of the same lineage. If the stimuli are discontinued, phenotypic traits could dissipate after the first or second generation. In other instances, longer lasting epigenetic changes adversely affect the phenotype of the third or fourth generation, often initiated by environmental factors adverse to human health that perpetuate aberrant patterns of transgenerational transmission of phenotype.

The purpose of this review is to simplify the enormous complexity of epigenetic biochemistry that links nuclear DNA to the environment. On one hand, the concept of epigenetics is relatively simple in that it describes a means by which genes are either turned on or off by a heritable epigenome. On the other hand, the environmental and biological controls that mediate these events are extraordinary in number, compounded by instances of similar methylation events that have opposite effects when occurring at different histone amino acids (e.g., H3K36me3 and H3K9me3) and by variation in the interpretation of studies performed in diverse organisms such as flies, plants, worms, yeast, ciliated protozoans, tumour cells and mammals.

Various mechanisms of epigenetic regulation: An overview

1. Genomic Imprinting
2. X – Chromosome inactivation
3. Bookmarking
4. Gene Silencing or Activation
5. Paramutation
6. Histone modification and heterochromatin regulation
7. Position effect and variegation
8. Teratogenic effects etc.

Mechanisms of Histone Modification Function

There are two characterized mechanisms for the function of modifications. The first is the disruption of contacts between nucleosomes in order to ‘‘unravel’’ chromatin and the second is the recruitment of nonhistone proteins. The second function is the most characterized to date. Thus, depending on the composition of modifications on a given histone, a set of proteins are encouraged to bind or are occluded from chromatin. These proteins carry with them enzymatic activities (e.g., remodelling ATPases) that further modify chromatin. The need to recruit an ordered series of enzymatic activities comes from the fact that the processes regulated by modifications(transcription, replication, repair) have several steps. Each one of these steps may require a distinct type of chromatin-remodelling activity and a different set of modifications to recruit them. Below is a more detailed description of the different mechanisms by which modifications work. Modifications may affect higher-order chromatin structure by affecting the contact between different histones in adjacent nucleosomes or the interaction of histones with DNA. Of all the known modifications, acetylation has the most potential to unfold chromatin since it neutralizes the basic charge of the lysine. This function is not easy to observe in vivo, but biophysical analysis indicates that intern-nucleosomal contacts are important for stabilization of higher-order chromatin structure. Thus, any alteration in histone charge will undoubtedly have structural consequences for the chromatin architecture. Furthermore, the recent development of strategies to make recombinant nucleosomes modified at specific sites has allowed this question to be addressed in vitro. By chemically ligating modified tail peptides onto recombinant histone core preparations, it has been possible to show that acetylation of H4K16 has a negative effect on the formation of a 30-nanometer fibre and the generation of higher-order structures. Phosphorylation is another modification that may well have important consequences for chromatin compaction via charge changes. The role of this modification has not been demonstrated rigorously in vitro but demonstrations of its role in mitosis, apoptosis, and gametogenesis are suggestive of such a role. Proteins are recruited to modifications and bind via specific domains. Methylation is recognized by chromo-like domains of the Royal family (chromo,tudor, MBT) and nonrelated PHD domains, acetylation is recognized by bromodomains, and phosphorylation is recognized by a domain within 14-3-3 proteins. A

number of proteins have been identified that are recruited to specific modifications. The recent isolation of several proteins that recognize H3K4me has highlighted the fact that their purpose is to tether enzymatic activities onto chromatin. BPTF, a component of the NURF chromatin-remodelling complex, recognizes H3K4me3 via a PHD domain. This recruitment tethers the SNF2L ATPase to activate H0XC8 gene expression. The PHD-finger proteinING2 tethers the repressive mSin3a-HDAC1 histone deacetylases complex to highly active, proliferation-specific genes after the exposure of cells to DNA-damaging agents. This finding represents a new mechanism of active shut-off of highly transcribed,H3K4-methylated genes. Two other H3K4mebindingproteins JMJD2A and CHD1 also tether enzymatic activities to chromatin, but in these instances the enzymatic activity resides within the methyl-binding protein:JMJD2A is a histone lysine demethylase that binds via a tudor domain and CHD1 is an ATPase that binds via a chromodomain. One other protein, WDR5, has been demonstrated to bind H3K4me1 and H3K4me2. however, structural analysis of this interaction does not support a purely methyl-recognition based interaction but suggests that this protein binds most avidly to the residues preceding H3K4 and in particular to H3R2. Perhaps this protein provides an adaptor function, augmenting the recognition of H3K4me.Proteins that bind other modified residues also deliver enzymes: H3K27me recruits the chromodomain containing polycomb protein PC2, which is associated with ubiquitin ligase activity specific for H2A; the chromocontainingHP1 protein binds H3K9me and is associated with deacetylase activity and methyltransferase activity. Equally important may be the effectiveness of histone modifications in preventing the docking of nonhistone proteins onto chromatin. The study of such pathways is less detailed, but examples include H3K4me disrupting the binding of the NuRD complex and H3T3ph preventing the binding of the INHAT complex. Both complexes have a repressive capability for transcription, so their occlusion by positively acting modifications makes sense. The abundance of modifications on the histone tail makes ‘‘crosstalk’’ between modifications very likely. Mechanistically such communication between modifications may occur at several different levels. Firstly, many different types of modification occur on lysine residues. This will undoubtedly result in some form of antagonism since distinct types of modifications on lysines are mutually exclusive. Secondly, the binding of a protein could be disrupted by an adjacent modification.

The best example of this being that of phosphorylation ofH3S10 affecting the binding of HP1 to methylated H3K9. Thirdly, the catalytic activity of an enzyme could be compromised by modification of its substrate recognition site; for example, isomerization ofH3P38 affects methylation of H3K36 by Set2. Fourthly, an enzyme could recognize its substrate more effectively in the context of a second modification; the example here is the GCN5 acetyltransferase, which may recognize H3 more effectively when it is phosphorylated at H3S10. Communication between modifications can also occur when the modifications are on different histone tails. The best studied example is the case of ubiquitinoylation of H2B being required for methylation of H3K4me3.

Histone code hypothesis:

The histone code is a hypothesis that the transcription of genetic information encoded in DNA is in part regulated by chemical modifications to histone proteins, primarily on their

unstructured ends that is in histone tails, particularly H3 and H4 by Acetylation, methylation, phosphorylation, ubiquitination etc. Together with similar modifications such as DNA methylation it is part of the epigenetic code. Histones associate with DNA to form nucleosomes, which themselves bundle to form chromatin fibres, which in turn make up the more familiar chromosome. Histones are globular proteins with a flexible N-terminus that protrudes from the nucleosome. Many of the histone tail modifications correlate very well to chromatin structure and both histone modification state and chromatin structure correlate well to gene expression levels. The critical concept of the histone code hypothesis is that the histone modifications serve to recruit other proteins by specific recognition of the modified histone via protein domains specialized for such purposes, rather than through simply stabilizing or destabilizing the interaction between histone and the underlying DNA. These recruited proteins then act to alter chromatin structure actively or to promote transcription.

Epigenetics in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae provides a well-studied model system for heritable silent chromatin, in which a nonhistone protein complex—the SIR complex—represses genes by spreading in a sequence-independent manner, much like heterochromatin in higher eukaryotes. The ability to study mutations in histones and to screen genome-wide for mutations that impair silencing has yielded an unparalleled depth of detail about this system. Recent advances in the biochemistry and structural biology of the SIR-chromatin complex bring us much closer to a molecular understanding of how Sir3 selectively recognizes the deacetylated histone H4 tail and demethylated histone H3 core. The existence of appropriate mutants has also shown how components of the silencing machinery affect physiological processes beyond transcriptional repression.

The fraction of chromatin in a eukaryotic nucleus that bears active genes is termed euchromatin. This chromatin condenses in mitosis to allow chromosomal segregation and decondenses in interphase of the cell cycle to allow transcription to occur. However, some chromosomal domains were observed by cytological criteria to remain condensed in interphase, and this constitutively compacted chromatin was called heterochromatin. With the development of new techniques, molecular rather than cytological features have been used to define this portion of the genome, and heterochromatin, which is often found at centromeres and telomeres, was shown to contain many thousands of simple repeat sequences, particularly in higher eukaryotic organisms. The repeat-rich genomic DNA tends to replicate late in S phase of the cell cycle, is found clustered at the nuclear periphery or near the nucleolus, and is resistant to nuclease attack. Importantly, the characteristic chromatin structure that is formed on repeat DNA tends to spread and repress nearby genes. In the case of the fruit fly locus *white*, a gene that determines red eye colour, epigenetic repression yields a red and white sectored eye through a phenomenon called position effect variegation (PEV). Mechanistically, PEV in flies reflects the recognition of methylated histone H3K9 by heterochromatin protein 1 (HP1), which can spread along the chromosomal arm. In *Saccharomyces cerevisiae*, also known as budding yeast, a distinct mechanism of heterochromatin formation has evolved, yet it achieves a very similar result.

S. cerevisiae is a microorganism commonly used for making beer and baking bread. However, unlike bacteria, it is a eukaryote. The chromosomes of budding yeast, like those of more complex eukaryotes, are bound by histones, enclosed in a nucleus and replicated from multiple origins during S phase. Still, the yeast genome is tiny with only 14 megabase pairs of genomic DNA divided among its 16 chromosomes, some not much larger than a bacteriophage genome. There are approximately 6000 genes in the yeast genome, closely packed along chromosomal arms, generally with less than 2 kb spacing between them. The vast majority of yeast genes are in an open chromatin state, meaning that they are either actively transcribed or can be rapidly induced. This, coupled with a very limited amount of simple repeat DNA, makes the detection of heterochromatin by cytological techniques very difficult in yeast.

Nonetheless, budding yeast has distinct heterochromatin-like regions adjacent to all 32 telomeres and at two silent mating loci on chromosome III, shown using molecular tools. Transcriptional repression at telomeres and the silent mating loci can spread into adjacent DNA and repression of the silent mating loci is essential for maintaining a mating-competent haploid state. Both the subtelomeric regions and the silent mating type loci repress integrated reporter genes in a position-dependent, epigenetic manner; they replicate late in S phase and are present at the nuclear periphery. Thus, these loci bear most of the functional characteristics of heterochromatin, without having cytologically visible condensation in interphase. By exploiting the advantages afforded by the small genome of yeast and its powerful genetic and biochemical tools, many basic principles of chromatin-mediated repression that are relevant to heterochromatin in more complex organisms have been discovered. Nonetheless, silent chromatin in budding yeast is dependent on a unique set of nonhistone proteins that do not deposit nor recognize histone H3 lysine 9 methylation.

Position-Effect Variegation, Heterochromatin Formation, and Gene Silencing in *Drosophila*

Position-effect variegation (PEV) results when a gene normally in euchromatin is juxtaposed with heterochromatin by rearrangement or transposition. When heterochromatin packaging spreads across the heterochromatin/euchromatin border, it causes transcriptional silencing in a stochastic pattern. PEV is intensely studied in *Drosophila* using the *white* gene. Screens for dominant mutations that suppress or enhance *white* variegation have identified many conserved epigenetic factors, including the histone H3 lysine methyltransferase SU(VAR). Heterochromatin protein HP1a binds H3K9me2/3 and interacts with SU(VAR), creating a core memory system. Genetic, molecular, and biochemical analysis of PEV in *Drosophila* has contributed many key findings concerning establishment and maintenance of heterochromatin with concomitant gene silencing.

Genes that are abnormally juxtaposed with heterochromatin, either by rearrangement or transposition, show a variegating phenotype. This is a result of the gene being silenced in some of the cells in which it is normally active. Because the change is caused by a change in the position of the gene in the genome, rather than a change in the gene itself, this phenomenon is termed “position-effect variegation” (PEV). The silencing that occurs in PEV can be attributed to the packaging of the reporter gene in a heterochromatic form, indicating that endogenous

heterochromatin formation, once initiated, can spread to encompass nearby genes. Genetic, cytological, and biochemical analyses are all possible in *Drosophila melanogaster*. In this article we will show how these different approaches have converged to identify many contributors to this system, leading to characterization of both structural proteins and modifying enzymes that play key roles in establishing and maintaining heterochromatin.

Heterochromatin formation depends critically on methylation of histone H3 at lysine 9 (H3K9me2/3), with concomitant association of heterochromatin protein 1 (HP1a) and other interacting proteins, including H3K9 methyltransferases (HKMTs); the multiple interactions of these proteins are required for the spreading and maintenance of heterochromatin. Targeting of heterochromatin formation to particular regions of the genome appears to involve multiple mechanisms, from satellite DNA-specific binding proteins to utilization of the RNA interference (RNAi) machinery. Although heterochromatic regions (pericentric regions, telomeres, the Y chromosome, and the small fourth chromosome) share a common biochemistry, each is distinct, and each is complex in different ways. Heterochromatin in *Drosophila* is gene poor, but it is not devoid of genes, and counterintuitively, those genes that reside in heterochromatin are often dependent on this environment for full expression. A complete understanding of heterochromatin formation and maintenance (including targeting and spreading) will need to include an explanation for the varying responses of different genes to this chromatin environment.

Probable Questions:

1. Define epigenetics. How it evolve?
2. Explain histone code hypothesis.
3. How epigenetics affect *Saccharomyces cerevisiae*?
4. Explain Position-Effect Variegation.
5. How epigenetics causes gene silencing in *Drosophla* ?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

Unit-II

Fungal models for epigenetic research: *Schizosaccharomyces pombe* and *Neurospora crassa*; RNAi and heterochromatin assembly, role of noncoding RNAs; epigenetic regulation in plants

Objective: In this unit we will discuss epigenetics of fungal models such as *Schizosaccharomyces pombe* and *Neurospora crassa*. We will also discuss epigenetic regulation in plants. RNAi and heterochromatin assembly as well as role of non coding RNAs will also e discussed.

A. Epigenetic study in *Schizosaccharomyces pombe*:

Assembly of heterochromatin, a dense chromatin structure that represses the expression of embedded genes, is vital for the establishment and maintenance of cell identity. A hallmark of heterochromatin is methylation of histone H3 at Lys-9 (H3K9me), a modification that is conserved from the fission yeast *Schizosaccharomyces pombe* to humans. Studies using *S. pombe* as a model organism have established the concept that the RNA interference (RNAi) pathway contributes to the assembly of heterochromatin. In fission yeast, the RNAi pathway is required predominantly at the pericentromeric regions, while the pathway is dispensable for the maintenance of the heterochromatin assembled at the subtelomeric regions and the mating-type locus. Notably, defects in the RNAi pathway lead to great loss of H3K9me and derepression of silencing at the pericentromeric regions but not at the subtelomeric regions or the mating-type locus. These regional differences in dependence on the RNAi pathway have provided researchers with clues to ascertain whether the factors of interest act specifically in the RNAi pathway or act more generally in the assembly of heterochromatin.

In the *S. pombe* RNAi pathway, formation of the small interfering RNA (siRNA)-containing effector complex is coupled to heterochromatin assembly. siRNA is generated, by the Dicer family endoribonuclease Dcr1, from double-stranded non-coding RNA that is complementary to heterochromatin. The siRNA duplex is loaded onto a non-chromatin-associated complex called Argonaute small interfering RNA chaperone (ARC), which contains the Argonaute family endoribonuclease Ago1. The loading of the siRNA duplex onto the Ago1 subunit requires the two ARC-specific subunits Arb1 and Arb2, which also inhibit the release of the passenger strand. This complex then changes its subunit composition to form a chromatin-associated effector complex called RNA-induced transcriptional silencing (RITS). The RITS complex is composed of Ago1, now binding single-stranded siRNA as a guide for target recognition, and the two RITS-specific subunits Chp1 and Tas3. Chp1 uses a chromodomain to recognize H3K9me , whereas Tas3 bridges Ago1 and Chp1.

With the ability to interact with both H3K9me and target RNA, RITS plays a central role in the self-enforcing cycle of RNAi-dependent heterochromatin assembly. RITS' function depends on two major interactions. On the one hand, RITS interacts with the RNA-dependent RNA

polymerase complex, which synthesizes double-stranded RNA for secondary siRNA generation. On the other hand, RITS interacts (via bridging by the linker protein Stc1) with the Clr4 histone methyltransferase-containing complex that methylates the H3 histone to create the H3K9me epigenetic marker. Thus, the formation of RITS is crucial for the generation of siRNA and for the assembly of RNAi-dependent heterochromatin. The formation of small RNA-containing effector complexes is generally assisted by heat-shock molecular chaperones. However, the heat-shock molecular chaperones responsible for the RNAi-dependent heterochromatin assembly remain unidentified. The candidates may belong to one or more of the distinct families of heat-shock proteins 40, 70, and 90 (Hsp40, Hsp70, and Hsp90, respectively).

Among the three Hsp families, the proteins belonging to the Hsp90 family promote the in vitro formation of small RNA-containing complexes in all species that have been tested. Notably, however, Hsp90-family proteins appear to act in species-specific manners. For example, the steps that require ATP hydrolysis by Hsp90-family proteins appear to differ among various species. For instance, Hsp90-mediated ATP hydrolysis is required for siRNA duplex loading in animal cells, but is instead required for passenger strand removal in plant cells. Similarly, the formation of small RNA-containing complexes does not necessarily require Hsp70-family proteins. An Hsp70 protein is essential for complex formation in the fruit fly *Drosophila melanogaster*, but not in the ciliated protozoan *Tetrahymena thermophila*. Therefore, the differences between species should be acknowledged in examining how such chaperones act in RNAi-dependent heterochromatin assembly.

The *S. pombe* genome encodes six Hsp70 proteins. These Hsps show high sequence similarity to their counterparts in the budding yeast *Saccharomyces cerevisiae*, where the cellular roles of Hsp70 s have been thoroughly examined. Among the six S. pombe Hsp70 proteins, Ssa1 and Ssa2, which show high sequence similarity to each other (identity: 94%), are recognized as nucleocytoplasmic Hsp70 protein. Ssa1 and Ssa2 also exhibit the strongest sequence similarity to the *D. melanogaster* Hsp70 protein Hsc70-4 (identity: 75% each), which is essential for the formation of a small RNA-containing complex in that organism.

The *S. pombe* genome encodes 26 Hsp40 family proteins, all of which harbour a characteristic DnaJ domain. These Hsp40 proteins can be divided into three classes: types I, II, and III. Type-I proteins are also found in *S. cerevisiae* and have the same names in the two yeast species. Mdj1 and Scj1 localize in mitochondria and the lumen of the ER, respectively. In contrast, Mas5 (also known as Ydj1 in *S. cerevisiae*) and Xdj1 localize in the cytosol and nucleus and are categorized as nucleocytoplasmic type-I Hsp40 proteins. Among the 26 Hsp40 proteins in S. pombe, Mas5 shows the greatest sequence similarity to the *D. melanogaster* protein Droj2 (identity: 41%), a protein that promotes the formation of a small RNA-containing complex in vitro.

RNAi and the RNA Pol II Machinery in Heterochromatin Assembly in *S. pombe* :

RNAi is an important mechanism contributing to heterochromatin formation in *S. pombe*. The phenomenon of RNAi was first discovered in Caenorhabditis elegans in which the expression of double-stranded RNA (dsRNA) abolished the expression of a homologous gene. It soon

became apparent that this form of RNAi is related to the process of transcriptional gene silencing (TGS) described in plants and quelling in *N. crassa*. These are processes of silencing that occur when a region is transcriptionally active, and transcripts that generate regions of dsRNA (e.g., through the self-annealing of inverted repeats) can be processed into small RNA fragments (termed small RNA biogenesis). These small RNAs are taken up by effector complexes and can trigger silent chromatin via targeting activities, which cause DNA methylation and histone modification. This process of silencing appears to be in operation from *S. pombe* to plants and metazoans, including mammals. Studies of the components of the RNAi machinery in *S. pombe* have led to significant advances in our understanding of RNAi-mediated chromatin modification and silencing. Mutants in the RNAi machinery in *S. pombe* result in reduced H3K9me2 and loss of silencing over the outer repeats of centromeres. Surprisingly at the time, these RNAi mutants revealed overlapping noncoding RNA (ncRNA) transcripts of a discrete size, originating from centromeric outer repeats. These ncRNAs were homologous to naturally occurring small dsRNAs called small interfering RNAs (siRNAs; 21 nt) that had been isolated and sequenced from S. pombe. These long noncoding double-stranded centromere repeat transcripts are cleaved by the Dicer (Dcr1) enzyme to generate siRNAs. These siRNAs then act to guide the RNAi machinery to homologous transcripts. Mutation of either subunit of RNA Pol II (Rpb2 and Rpb7) results in defective centromere silencing although these mutations display very different phenotypes. The rpb7-1 mutant shows reduced levels of centromere repeat transcription, resulting in less ncRNA and, consequently, less siRNA production and a loss of silent chromatin. This implies that RNA Pol II is required for the transcription of centromere repeats, which then provides the primary substrate for RNAi. In contrast, centromeric transcripts in the rpb2- m203 mutant are produced but not processed into siRNA, and H3K9 methylation at centromeres is reduced. These studies indicate that RNAi not only requires an RNA Pol II transcript but that, like other RNA-processing events, the production of centromeric siRNA may be coupled to transcription by interactions between the RNAi machinery, chromatin, histone-modifying enzymes, and RNA Pol II. The RNAi machinery in S. pombe is complex and not yet fully understood. In addition to transcription of noncoding centromeric outer repeats by RNA Pol II and the processing of transcripts into siRNAs by Dcr1, the key activities of the RNAi machinery involve two complexes: RITS and RDRC (RNA-directed RNA polymerase complex). The RITS complex incorporates siRNAs to then direct it to centromere outer repeats via sequence recognition as well as H3K9me2/3 recognition through the Chp1 chromodomain. RDRC is recruited to amplify the process of TGS by generating more long double stranded ncRNAs through the action of Rdp1 (RNA-directed RNA polymerase 1). Rdp1 transcribes from transcripts primed with siRNAs presented by the RITS complex. The chromatin-modifying machineries that execute chromatin changes include the CLRC (for Clr4-Rik1-Cul4 complex) and the SHREC complex. CLRC is recruited by the RITS complex via the Stc1 protein. Once recruited, Clr4 methylates H3K9 over the outer repeats and this allows it to bind directly to H3K9me2/3 via its chromodomain. The HP1 homologs Swi6 and Chp2 play further roles in establishing and maintaining heterochromatin. Initiation of transcription, transcriptional elongation, and transcript processing are as important for heterochromatin assembly as they are for euchromatic gene expression. Several associated factors and activities are important for these different steps of the RNA Pol II transcription cycle, in addition to RNA Pol II itself. FACT, an RNA Pol II-

associated chromatin assembly factor, and Spt6, another RNA Pol II-associated protein both colocalize to pericentric repeats. Spt6 is specifically required for facilitating trimethylation of H3K9, Swi6 binding, siRNA production, and recruitment of the HDAC enzyme Clr3. Mutations in the FACT component, Pob3, have a similar phenotype to spt6 implicating FACT in the same processes. Interestingly, mutations in chromatin-modifying activities or RNA-processing factors have been shown to suppress the need for Dcr1. For example, loss of Mst2 activity, a H3K14-specific acetyltransferase, completely suppresses dcr1 mutants; that is, it eliminates the need for the RNAi machinery in heterochromatin maintenance, but not in the establishment of new heterochromatin. This suggests that an important role of the RNAi directed heterochromatin process is to prevent Mst2 activity, which might interfere with CLRC recruitment. Another example of bypassing the RNAi pathway is by knocking out the gene encoding Mlo3, involved in mRNP biogenesis and RNA quality control. This also suppresses the need for Dcr1. It was suggested that in mlo3 ago1 knockout cells there is an aberrant accumulation of centromeric transcripts, which results in the recruitment of the CLRC complex via Rik1. In wild-type cells, CLRC acts downstream from the RNAi pathway and is required for heterochromatin silencing by recruiting and promoting the activity of the Clr4 enzyme. Thus, this RNAi-independent recruitment mechanism of CLRC allows the induction of heterochromatin assembly at repeat sequences in mlo3 ago1 knockout cells. Another RNA Pol II linked event is splicing of pre mRNA by the spliceosome. There is not a general requirement for the spliceosome or the splicing process in heterochromatin formation, although some specific splicing factors are required for siRNA production. It remains to be determined how exactly these splicing factors contribute to RNAi, but their physical interaction with the RDRC suggests a direct role in RNAi.

B. Epigenetic study in *Neurospora crassa*:

The filamentous fungus *Neurospora crassa* has provided a rich source of knowledge on epigenetic phenomena that would have been difficult or impossible to gain from other systems. *Neurospora* sports features found in higher eukaryotes but absent in both budding and fission yeast, including DNA methylation and H3K27 methylation, and also has distinct RNA interference (RNAi)-based silencing mechanisms operating in mitotic and meiotic cells. This has provided an unexpected wealth of information on gene silencing systems. One silencing mechanism, named repeat-induced point mutation (RIP), has both epigenetic and genetic aspects and provided the first example of a homology-based genome defence system. A second silencing mechanism, named quelling, is an RNAi-based mechanism that results in silencing of transgenes and their native homologs. A third, named meiotic silencing, is also RNAi-based but is distinct from quelling in its time of action, targets, and apparent purpose.

Fungi provide excellent models for understanding the structure and function of chromatin both in actively transcribed regions (euchromatin) and in transcriptionally silent regions (heterochromatin). The budding yeast, *Saccharomyces cerevisiae*, has been an invaluable eukaryotic model for studying chromatin structure associated with transcription at euchromatic regions and providing a paradigm for silent chromatin. The fission yeast, *Schizosaccharomyces pombe*, has some epigenetic machinery that is absent from *S. cerevisiae* but common in higher

organisms—most notably for RNA interference (RNAi) and for methylation of lysine 9 of histone H3 (H3K9me). Research using *S. pombe* has provided invaluable information on the structure and function of heterochromatin, principally found in regions of the centromeres, telomeres, and silent mating-type genes. This article focuses on a third model system, namely the filamentous fungus *Neurospora crassa*. Although not as commonly studied as the yeasts, *Neurospora* has proved to be a remarkably rich source of knowledge that would have been difficult or impossible to gain from other systems. *Neurospora* sports features found in higher eukaryotes, including DNA methylation and the H3K27 methylation ("Polycomb") system that both budding and fission yeasts lack, as well as RNAi and other epigenetic processes found in the yeasts. This has provided an unexpected wealth of information on gene silencing systems, some of which operate at distinct stages of its life cycle. The first such mechanism, named repeat-induced point mutation (RIP), has both epigenetic and genetic aspects and provided the first example of a homology-based genome defence system. The second, named quelling, is an RNAi-based mechanism that results in silencing of transgenes and their native homologs. The third, named meiotic silencing (or meiotic silencing by unpaired DNA), is also RNAi-based but is distinct from quelling in its time of action, targets, and apparent purpose. Although we are still in the early days of epigenetic studies in all organisms, it is already clear that yeasts and filamentous fungi such as *N. crassa* will continue to serve as rich sources of information on epigenetic mechanisms operative in a broad range of eukaryotes.

RNAi and Heterochromatin Assembly :

The intersection between RNA interference (RNAi) and heterochromatin formation has brought together two areas of gene regulation that had previously been thought to operate by different, perhaps even unrelated, mechanisms. Heterochromatin was originally defined nearly 80 years ago using cytological staining methods as those chromosome regions that retain a condensed appearance throughout the cell cycle. Early investigators studying the relationship between chromosome structure and gene expression noticed that certain chromosome rearrangements resulted in the spreading of heterochromatin into adjacent genes, which then became silent. But, the seemingly stochastic patterns of spreading gave rise to genetically identical populations of cells that had different phenotypes. This phenomenon, initially described in *Drosophila* as position-effect variegation, provides a striking example of epigenetic regulation. The term RNAi was first used to describe gene silencing that resulted from the introduction of homologous antisense or double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans*. But, it was soon recognized that a related mechanism involving RNA accounted for posttranscriptional transgene silencing (PTGS) described earlier in petunia and tobacco. In contrast, heterochromatin was widely believed to operate directly at the chromatin level to cause transcriptional repression by a mechanism referred to as transcriptional gene silencing (TGS). This article focuses on the relationship between the RNAi pathway and the formation of epigenetically heritable heterochromatin at specific chromosome regions. It draws on recent examples that show this relationship in the fission yeast *Schizosaccharomyces pombe* and the mustard plant *Arabidopsis thaliana*.

The fission yeast nuclear genome is composed of three chromosomes that range in size from 3.5 to 5.7 Mb. Each chromosome contains large blocks of repetitive DNA, particularly at

centromeres, which are packaged into heterochromatin. The mating-type loci (which control cell type) and subtelomeric DNA regions also contain repetitive sequences that are packaged into heterochromatin. We now know that the assembly of DNA into heterochromatin plays both regulatory and structural roles. In the case of the mating-type loci in yeast, regulation of gene transcription by heterochromatin is important for cell-type identity. In the case of telomeres and centromeres, heterochromatin plays a structural role that is important for proper chromosome segregation during cell division. Moreover, repetitive DNA sequences and transposable elements account for a large fraction, in some cases more than half, of the genomes of many eukaryotic cells. Heterochromatin and associated mechanisms play a critical role in regulating the activity of repeated sequences, thus maintaining genome stability.

Recent studies have uncovered a surprising requirement for components of the RNAi pathway in the process of heterochromatin formation in fission yeast and have provided insight into how these two pathways can work together at the chromatin level. Briefly, small interfering RNA (siRNA) molecules and their Argonaute-binding proteins assemble into the RNA-induced transcriptional silencing (RITS) complex and direct epigenetic chromatin modifications and heterochromatin formation at complementary chromosome regions. RITS uses siRNA-dependent base pairing to guide association with nascent RNA sequences at the target locus destined to be silenced, an association that is stabilized by direct binding to methylated histone H3 at lysine (K)9 (H3K9me). The presence of these two activities in RITS (i.e., siRNA base-pairing and association with chromatin via methylated H3K9) triggers heterochromatin formation in concert with well-known heterochromatin-associated factors, and RNA polymerase II (Pol II) directly linking RNA silencing to heterochromatin modification and silencing.

In *A. thaliana* and many other eukaryotes, repeat sequences such as retroelements and other transposons are targeted for inactivation at the chromatin level by mechanisms that couple small RNA-mediated targeting with histone H3K9, but also DNA methylation. Although the existence of a RITS complex is not always clear, components of the RNAi and related pathways are required for the initiation and maintenance of these repressive methylation events, along with Pol II and related polymerases. In this article, we will discuss how heterochromatic siRNAs are produced, and how they mediate DNA and/or chromatin modifications in fission yeast and *A. thaliana*.

Role of Non Coding RNA

Non-coding RNA (ncRNA) is a functional RNA molecule that is transcribed from DNA but not translated into proteins.

Epigenetic related ncRNAs include miRNA, siRNA, piRNA and lncRNA. In general, ncRNAs function to regulate gene expression at the transcriptional and post-transcriptional level. Those ncRNAs that appear to be involved in epigenetic processes can be divided into two main groups; the short ncRNAs (<30 nts) and the long ncRNAs (>200 nts). The three major classes of short non-coding RNAs are microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). Both major groups are shown to play a role

in heterochromatin formation, **histone modification**, **DNA methylation** targeting, and gene silencing.

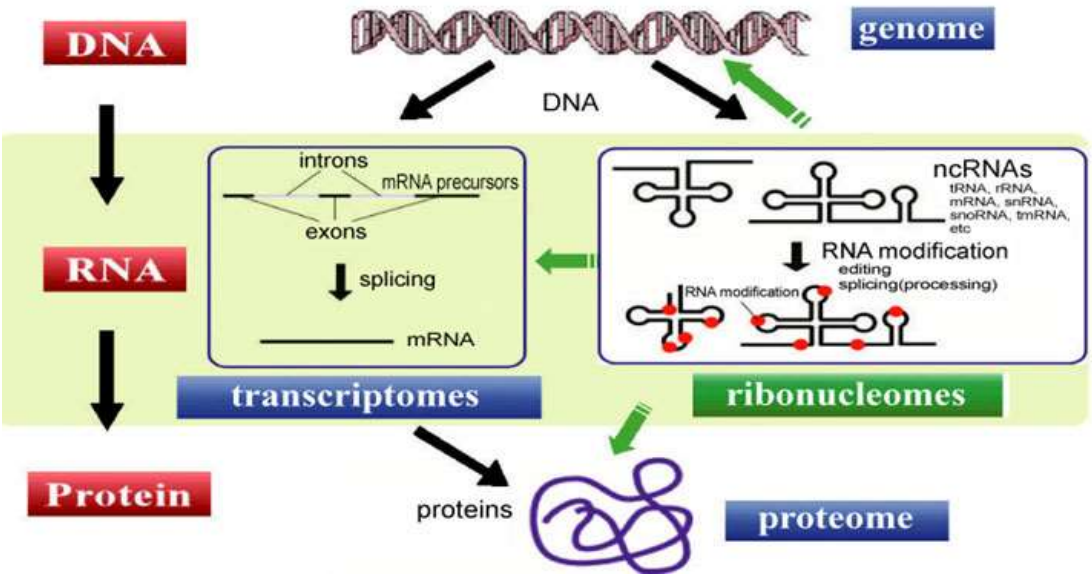


Fig: In vivo function of NcRNA in living organisms

Short ncRNAs

MicroRNAs (miRNA) generally bind to a specific target messenger RNA with a complementary sequence to induce cleavage, or degradation or block translation. This may be done in the context of a feedback mechanism that involves chromosome methylation. For example, miRNA genes mir-127 and mir-136 were found to be involved in regulating the genetic imprinting of Rtl1, a key gene involved in placenta formation in mice. Methylation of a specific region in the paternal chromosome results in expression of Rtl1. If the chromosome is not methylated, as on the maternal chromosome, mir-127 and mir-136 are produced and bind to the Rtl1 transcript and induce degradation. Lack of Rtl1 protein expression due to improper epigenetic modifications can result in foetal death in mice.

Short interfering RNAs (siRNA) function in a similar way as miRNAs to mediate post-transcriptional gene silencing (PTGS) as a result of mRNA degradation. In addition to this function, siRNAs have also been shown to induce heterochromatin formation via an RNA-induced transcriptional silencing (RITS) complex which when bound to siRNA promotes H3K9 methylation and chromatin condensation.

Piwi-interacting RNAs (piRNA) are so named due to their interaction with the piwi family of proteins. The primary function of these RNA molecules involves chromatin regulation and suppression of transposon activity in germline and somatic cells. PiRNAs that are antisense to expressed transposons target and cleave the transposon in complexes with PIWI-proteins. This cleavage generates additional piRNAs which target and cleave additional transposons. This cycle continues to produce an abundance of piRNAs and augment transposon silencing.

Long ncRNAs

Many lncRNAs can complex with chromatin-modifying proteins and recruit their catalytic activity to specific sites in the genome, thereby modifying chromatin states and influencing gene expression. The majority of non-coding RNA transcripts belong to the group lncRNAs. Long ncRNAs function in chromatin remodelling, transcriptional regulation, post-transcriptional regulation, and as precursors for siRNAs.**7** One particular subgroup of lncRNAs, the large intergenic non-coding RNAs (lincRNAs), has been associated with chromatin modifying complexes which can target specific genomic loci to promote specific epigenetic states. One widely known example of this is the role of X-inactive specific transcript gene (Xist), in X-chromosome inactivation (XCI). This process involves two lncRNAs; Xist and its antisense transcript Tsix, a negative regulator of Xist. Prior to differentiation, Xist and Tsix are actively transcribed due to H3K4 dimethylation of the Xist gene. In this state XCI is a random event. Upon differentiation, Xist expression is elevated resulting in Xist RNA coating the future inactive X chromosome which triggers extensive histone methylation and chromosome inactivation.

Epigenetic Regulation in Plants

The study of epigenetics in plants has a long and rich history, from initial descriptions of non-Mendelian gene behaviours to seminal discoveries of chromatin-modifying proteins and RNAs that mediate gene silencing in most eukaryotes, including humans. Genetic screens in the model plant *Arabidopsis* have been particularly rewarding, identifying more than 130 epigenetic regulators thus far. The diversity of epigenetic pathways in plants is remarkable, presumably contributing to the phenotypic plasticity of plant postembryonic development and the ability to survive and reproduce in unpredictable environments.

Plants are masters of epigenetic regulation. All of the major epigenetic mechanisms known to occur in eukaryotes are used by plants, with the responsible pathways elaborated to a degree that is unsurpassed in other taxa. DNA methylation occurs in CG, CHG, and CHH sequence contexts in plant genomes, in patterns that reflect a balance between enzyme activities that install, maintain, or remove methylation. As in other eukaryotes, histone-modifying enzymes influence epigenetic states in plants and these enzymes are encoded by comparatively large gene families, allowing for diversified as well as overlapping functions. RNA-mediated gene silencing is accomplished using multiple distinct pathways to combat viruses, tame transposons, orchestrate development, and help organize the genome. The interplay between DNA methylation, histone modification, and noncoding RNAs provides plants with a multi-layered and robust epigenetic circuitry.

The prominence of epigenetic regulation in plants reflects their mode of development, lifestyle, and evolutionary history. Unlike growth in mammals, in which organ and tissue formation is largely specified during embryonic development, plants grow by continuously producing new organs from self-sustaining stem cell populations known as meristems. Consequently, postembryonic development in plants is a continuous process shaped by environmental influences resulting in a high degree of phenotypic plasticity. Because plants are unable to

escape their surroundings, they are forced to cope with changeable and often unfavorable growth conditions. Epigenetic regulatory mechanisms can facilitate metastable changes in gene activity and fine-tune gene expression patterns, thus enabling plants to survive and reproduce successfully in unpredictable environments. Polyploidization, an increase in the number of sets of chromosomes, is common in plants, amplifying gene families and fostering the functional specialization of duplicated genes, including those involved in epigenetic regulation.

Understanding the epigenetic regulatory machinery of plants has come, in large part, from genetic screens, most notably in *Arabidopsis thaliana*, a member of the mustard family that is highly amenable to genetic analyses and was the first plant species to have its genome sequenced. Crop plants, particularly maize, have also contributed substantially to the discovery of epigenetic phenomena and epigenetic regulatory mechanisms. The study of plant epigenetics and epigenomics has a long and rich history and, in synergy with parallel studies in animal and fungal systems, is contributing significantly to our basic understanding of epigenetic regulation.

Probable Questions:

1. How epigenetics affect *Schizosaccharomyces pombe* ?
2. What is the role of RNAi and RNA Pol II Machinery in Heterochromatin Assembly in *S. pombe ?*
3. Describe different types of short non coding RNAs .
4. Describe different types of long non coding RNAs .
5. How plants are affected by epigenetics?
6. How epigenetics affect *Neurospora crassa* ?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

Unit-III

Chromatin structure and epigenetics marks - transcriptional silencing by polycomb group proteins, transcriptional regulation by trithorax group proteins, histone variants and epigenetics, epigenetic regulation of chromosome inheritance

Objective: In this unit we will discuss Chromatin structure and epigenetics marks - transcriptional silencing by polycomb group proteins , transcriptional regulation by trithorax group proteins, histone variants and epigenetics, epigenetic regulation of chromosome inheritance

Underlying mechanism of Chromosomal epigenetic inheritance:

Based on of the nucleosome modifications that occur at the silent mating-type region in yeast, Thon and colleagues developed a mathematical model of histone PTM regulation in the context of a two state process. The authors assumed that the model is applicable for bi-stable conditions meaning that transition from one state to another occurs but at a relatively low frequency. The bi-stability is efficiently maintained through replication assuming that histone PTMs are randomly segregated during replication and that newly deposited nucleosomes are naïve (i.e. devoid of the histone modifications regulating the transition from one state to another and are therefore potential targets for these modifications). We mentioned in the previous section that newly incorporated histones do not carry the PTMs potentially involved in transmitting epigenetic information. The bi-stability requires cooperativity between the histone modifications and positive feedback for the spreading of a defined mark. Furthermore this positive feedback should propagate the mark not only linearly, i.e. to adjacent nucleosomes, but also reach targets several nucleosomes away, thus highlighting the importance of higher order chromatin structure. In the next sections, we will consider known mechanisms that could be critical for the propagation of epigenetic information in light of these theoretical predictions, which we believe to be of great importance in understanding transmission of histone marks and the establishment of chromatin domains that are inherited.

Importantly, while we have some idea about the propagation of DNA methylation and models are emerging for the duplication of histone PTMs, very little is known about inheritance of nucleosome occupancy or histone variants. It was reported that the silencing of

the *MLH1* promoter in cancer is associated with specific changes in nucleosome occupancy. Considering that this promoter is also specifically DNA methylated in cancer cells and that changes in nucleosome occupancy are reversed by inhibition of DNA methylation, nucleosome occupancy in this case might be regulated by DNA methylation. Regarding histone variants, a better characterization of the histone deposition machinery will be required to understand how they might be stably maintained at a defined locus.

Polycomb Group (PcG) of Proteins

What are PcG proteins?

Polycomb-group (PcG) genes encode chromatin proteins involved in stable and heritable transcriptional silencing. PcG proteins participate in distinct multimeric complexes that deposit, or bind to, specific histone modifications (e.g., H3K27me3 and H2AK119ub1) to prevent gene activation and maintain repressed chromatin domains. PcG proteins are evolutionary conserved and play a role in processes ranging from vernalization and seed development in plants, over X-chromosome inactivation in mammals, to the maintenance of stem cell identity. PcG silencing is medically relevant as it is often observed in human disorders, including cancer, and tissue regeneration, which involve the reprogramming of PcG-controlled target genes.

Brief idea about transcriptional memory

Organs of humans, animals, and plants are constructed from a large pool of distinct cell types, each performing a specialized physiological or structural function. With very few exceptions, all cell types contain the same genetic information encoded in their DNA. Thus, the distinctiveness of a given cell type is achieved through specific gene expression programs. As a consequence, cell lineages need to have these programs maintained during growth and cell division. This implies the existence of a memory system that ensures the faithful transmission of information (i.e., which gene is active or repressed) from mother to daughter cells. The existence of such a system is illustrated by the fact that cultured tissues of plants and animals usually maintain their differentiated characters even if grown in a foreign environment. By way of example, ivy plants regenerated after tissue culture produce the type of leaf corresponding to the phase of development from which the original tissue was taken (i.e., juvenile or adult leaf).

Brief idea on the roles and effects of PcG proteins

The major question to be addressed here concerns the molecular identity of factors contributing to the mechanism(s) of "cellular" or "transcriptional memory," which maintains a determined state over many cell divisions. Genetic analyses in *Drosophila melanogaster* have identified regulators crucial in maintaining the morphology of individual body segments that are determined by the action of the HOX genes. In *Drosophila* males, the first thoracic segment has legs with sex combs. Legs on the second and third thoracic segment lack these structures. In the 1940s, *Drosophila* mutants were identified (Polycomb and extra sex combs) wherein males had sex combs on all legs. These morphological alterations reflect homeotic transformations of the second and third leg identities into the first leg identity. Subsequent molecular studies showed that these mutations did not affect the products of the HOX genes themselves, but rather the way HOX gene activity was spatially controlled. Throughout the years, a large number of similar regulatory genes were identified, and were classified into two antagonistic groups: the Polycomb (PcG) and Trithorax (TrxG) group. Whereas the PcG proteins are required to maintain the silenced state of developmental regulators such as the HOX genes, the TrxG proteins are generally involved in maintaining the active state of gene expression. Thus PcG and TrxG proteins embody the molecular components of cellular memory.

Proteins of both groups form large multimeric protein complexes that act on their target genes by modulating chromatin structure. In *Drosophila,* it was shown that transcription factors recruit PcG complexes to a DNA sequence called a PcG response element (PRE). Once recruited, they establish a silent chromatin state that can be inherited over many cell divisions. Members of PRC2 are highly conserved between plants and animals, whereas PRC1 proteins are less well conserved. This implies conservation, but also diversity, in the basic building blocks of the cellular memory system. In addition to the function of PcG complexes in the maintenance of cell types, they may also play important roles in stem cell plasticity and regeneration. Also, their deregulation can lead to neoplastic transformation and cancer. Thus, PcG proteins play a crucial role in many fundamental processes of normal development and disease in multicellular eukaryotes.

Transcriptional Regulation by Trithrorax group (trxG) of proteins

What are trxG proteins?

The trithorax group of genes (trxG) was identified in mutational screens that examined developmental phenotypes and suppression of *Polycomb* mutant phenotypes. The protein

products of these genes are primarily involved in gene activation, although some can also have repressive effects. There is no central function for these proteins. Some move nucleosomes about on the genome in an ATP-dependent manner, some covalently modify histones such as methylating lysine 4 of histone H3, and some directly interact with the transcription machinery or are a part of that machinery. It is interesting to consider why these specific members of large families of functionally related proteins have strong developmental phenotypes.

Brief idea about the roles and effects of trxG in Transcriptional Memory:

All cells in an organism must be able to "remember" what type of cell they are meant to be. This process, referred to as "cellular memory" or "transcriptional memory," requires two basic classes of mechanisms. The first class, functions to maintain an "off" state for genes that, if turned on, would specify an inappropriate cell type. The Polycomb-group (PcG) proteins have as their primary function a repressive role in cellular memory. The second class of mechanisms is composed of those that are required to maintain key genes in an "on" state. Any cell type requires the expression of master regulatory proteins that direct the specific functions required for that cell type. The genes that encode these master regulatory proteins must be maintained in an "on" state throughout the lifetime of an organism to maintain the proper cell types within that organism.

The proteins that are involved in maintaining the "on" state are called trithorax-group (trxG) proteins in honor of the trithorax gene, the founding member of this group of regulatory proteins. A large group of proteins with diverse functions make up the trxG. The roles these proteins play in the epigenetic mechanisms that maintain the "on" state appear more complex at this juncture than the roles for PcG proteins in repression. The first complexity is that a very large number of proteins and mechanisms are needed to actively transcribe RNA from any gene. Thus, in contrast to repression, which might be accomplished by comparatively simple mechanisms that block access of all proteins, activation of a gene requires numerous steps, any of which might play a role in maintaining an "on" state. Thus, there are numerous possible stages in which a trxG protein might work.

A second complexity in thinking about trxG proteins is that proteins that function in activation can also, in different contexts, function in repression. This might appear counterintuitive, but depending on the precise architecture of a gene, the same protein performing its function might in one case help a gene become activated, and in another case help a different gene become repressed. At this time it does not appear that trxG proteins are dedicated solely to the

maintenance of gene expression, but that these proteins can also play multiple roles in the cell. These complexities make for several interesting unanswered questions. Why are only some of the proteins needed to activate transcription also critical for maintenance of transcription? Do these proteins have functions that are uniquely suited to maintaining the active state? Or are some of these proteins needed for maintenance solely because an evolutionary accident that made them key regulators of gene(s) particularly important to development?

As we will see when we discuss mechanisms of action, some of the trxG proteins are involved in regulating chromatin structure in opposition to the mechanisms used by the PcG proteins. trxG proteins can place covalent posttranslational modifications (PTMs) on chromatin or can alter chromatin by changing the structure and position of the nucleosomes that are the building blocks of chromatin. Other trxG proteins function as part of the transcription machinery. Thus, these proteins are found in a wider variety of complexes than the PcG proteins and are likely to play more complicated roles in epigenetic mechanisms.

Numerous developmental decisions—including the determination of cell fates—are made in response to transient positional information in the early embryo. These decisions are dependent on changes in gene expression. This allows cells with identical genetic blueprints to acquire unique identities and follow distinct pathways of differentiation. The changes in gene expression underlying the determination of cell fates are heritable; a cell's fate rarely changes once it is determined, even after numerous cell divisions and lengthy periods of developmental time. Understanding the molecular mechanisms underlying the maintenance of the determined state has long been a goal of developmental and molecular biologists.

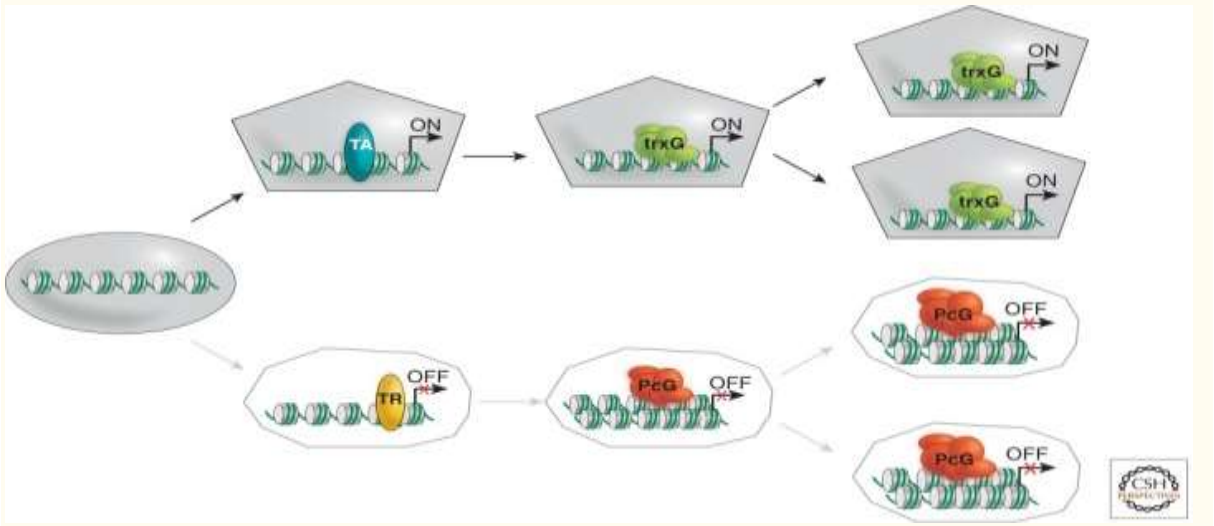


Figure 1.

The concept of cellular memory. Schematic illustration highlighting the role of trxG complexes in maintaining heritable states of active gene expression in contrast to heritable silencing by PcG complexes, as defined originally for the *Drosophila* homeotic (Hox) gene cluster.

Many of the regulatory proteins involved in the maintenance of heritable states of gene expression were identified in studies of *Drosophila* homeotic (Hox) genes. Hox genes encode homeodomain transcription factors that regulate the transcription of batteries of downstream target genes, which in turn specify the identities of body segments. In *Drosophila*, Hox genes are found in two gene complexes: the Antennapedia complex (ANT-C), which contains the Hox genes *labial* (*lab*), *Deformed*, *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*); and the bithorax complex, which contains the Hox genes *Ultrabithorax* (*Ubx*), *abdominalA* (*abdA*), and *AbdominalB* (*AbdB*;). Each Hox gene specifies the identity of a particular segment, or group of segments, along the anterior–posterior axis of the developing fly. For example, *Antp* specifies the identity of the second thoracic segment, including the second pair of legs, whereas *Ubx* specifies the identity of the third thoracic segment, including the balancer organs located behind the wings. Thus, the transcription factors encoded by Hox genes function as master regulatory switches that direct the choice between alternative pathways of development.

The transcription of Hox genes must be regulated precisely because dramatic alterations in cell fates can result from their inappropriate expression. For example, the derepression of *Antp* in head segments transforms antennae into legs, whereas the inactivation of *Ubx* in thoracic segments transforms balancer organs into wings. In *Drosophila*, the initial patterns of Hox transcription are established early in embryogenesis by transcription factors encoded by segmentation genes. The proteins encoded by segmentation genes—including the gap, pair-rule, and segment polarity genes—subdivide the early embryo into 14 identical segments. These proteins also establish the initial patterns of Hox transcription, the first step toward the development of segments with distinct identities and morphology. The majority of segmentation genes, however, are transiently expressed during early development. Once established, the segmentally restricted patterns of Hox transcription must be maintained throughout subsequent embryonic, larval, and pupal stages to maintain the identities of the

individual body segments. This function is performed by two other groups of regulatory proteins: the Polycomb group of repressors (PcG) and the trithorax group of transcriptional regulators (trxG). The regulation of Hox transcription therefore consists of at least two distinct phases: establishment (by segmentation genes) and maintenance (by PcG and trxG genes; Fig. 2).

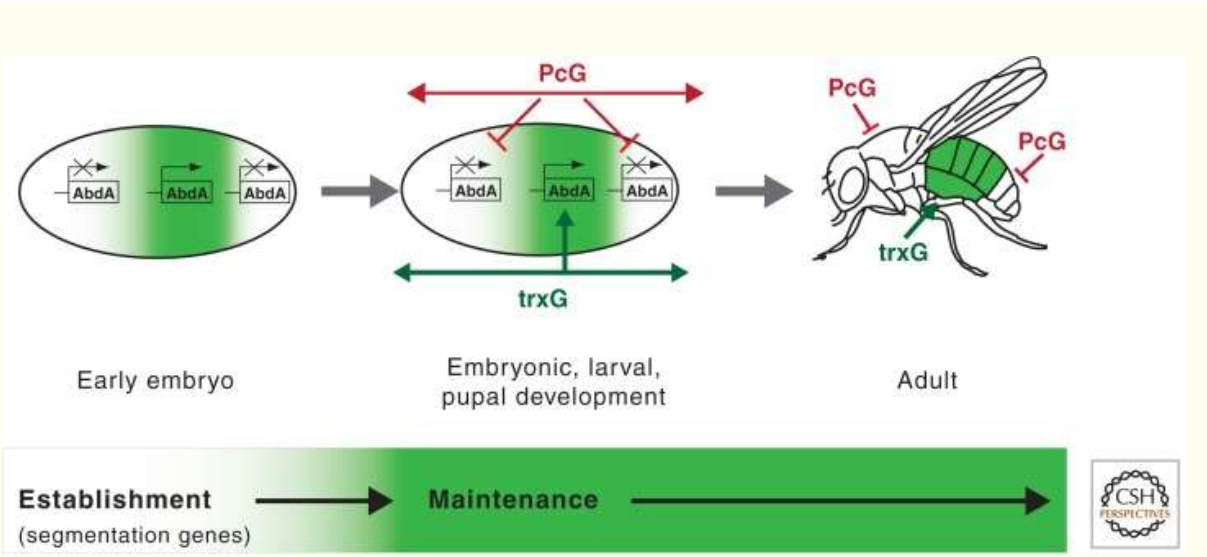


Figure 2.

Regulation of Hox transcription. The boundaries of *abd-A* transcription and other Hox genes are established by segmentation proteins. These include the products of gap and pair-rule genes, which subdivide the embryo into 14 identical segments. During subsequent development, the "off" or "on" states of Hox transcription are maintained by the ubiquitously expressed members of the trxG of activators and the PcG of repressors via mechanisms that remain poorly understood.

Histone Variants and Epigenetics

Histones package DNA by assembling into nucleosome core particles, whereas the double helix wraps around. Over evolutionary time, histone-fold domain proteins have diversified from archaeal ancestors into the four distinct subunits that comprise the familiar octamer of the eukaryotic nucleosome. Further diversification of histones into variants results in differentiation of chromatin that can have epigenetic consequences. Investigations into the evolution, structure, and metabolism of histone variants provides a foundation for understanding the participation of chromatin in important cellular processes and in epigenetic

memory. Most histones are synthesized at S phase for rapid deposition behind replication forks to fill in gaps resulting from the distribution of pre-existing histones. In addition, the replacement of canonical S-phase histones by variants, independent of replication, can potentially differentiate chromatin. The replacement of a canonical histone by a noncanonical variant is a dynamic process that changes the composition of chromatin.

The differentiation of chromatin by a histone variant is especially conspicuous at centromeres, in which the H3 variant, CENP-A, is assembled into specialized nucleosomes that form the foundation for kinetochore assembly. A centromeric H3 (cenH3) counterpart of CENP-A is found in all eukaryotes. In plants and animals, the faithful assembly of cenH3-containing nucleosomes at centromeres does not appear to require centromeric DNA sequences, a spectacular example of epigenetic inheritance. Some cenH3s have evolved adaptively in regions that contact DNA, which suggests that centromeres compete with each other, and cenH3s and other centromere-specific DNA-binding proteins have adapted in response. This process could account for the large size and complexity of centromeres in plants and animals.

Chromatin can also be differentiated outside of centromeres by incorporation of a constitutively expressed form of H3, called H3.3, which is the substrate for replication-independent nucleosome assembly. Replacement with H3.3 occurs at active genes, a dynamic process with potential epigenetic consequences. Differences between H3 and H3.3 in their complement of covalent modifications might underlie changes in the properties of chromatin at actively transcribed loci.

Several H2A variants can also differentiate or regulate chromatin. H2A.X is defined as a variant by a four-amino-acid carboxy-terminal motif whose serine residue is the site for phosphorylation at sites of DNA double-stranded breaks. Phosphorylation of H2A.X is an early event in double-strand break repair, in which it is thought to concentrate components of the repair machinery. H2A.X phosphorylation also marks the inactive XY bivalent during mammalian spermatogenesis and is required for condensation, pairing, and fertility.

H2A.Z is a structurally diverged variant that has long presented an enigma. Studies in yeast have implicated H2A.Z in establishing transcriptional competence and in counteracting heterochromatic silencing. The biochemical complex that replaces H2A with H2A.Z in nucleosomes is an ATP-dependent nucleosome remodeler, providing the first example of a specific function for a member of this diverse class of chromatin-associated machines.

Two vertebrate-specific variants, macroH2A and H2A.B (also called H2A.Bbd), display contrasting features when packaged into nucleosomes in vitro, with macroH2A impeding and H2A.B facilitating transcription. These features are consistent with their localization patterns on the epigenetically inactivated mammalian X chromosome: macroH2A showing enrichment and H2A.B showing depletion. The emerging view from these studies is that histone variants and the processes that deposit them into nucleosomes provide a primary differentiation of chromatin that might serve as the basis for epigenetic processes.

Probable questions:

1. What are PcG proteins?
2. Discuss Briefly about transcriptional memory.
3. Discuss roles and effects of PcG proteins.
4. What are trxG proteins?
5. Discuss roles and effects of trxG in Transcriptional Memory.
6. Discuss Histone Variants and Epigenetics in brief.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

Unit-IV

Epigenetic regulation of the X chromosomes in *C.elegans*, dosage compensation in *Drosophila*, dosage compensation in mammals; types mechanism of chromatin remodeling.

Objective: In this unit we will discuss about Epigenetic regulation of X chromosome, dosage compensation in human and Drosophila. We will also discuss types and mechanism of chromatin remodelling.

THE PHENOMENON OF DOSAGE COMPENSATION WAS DISCOVERED IN DROSOPHILA:

The karyotypes (i.e., ensemble of chromosomes) of many organisms include a pair of sex chromosomes. In Drosophila, females have two sex chromosomes called the X chromosomes that are identical in shape and genetic content; both X chromosomes are active in all somatic cells. Males have one X and a Y chromosome that differs from the X in morphology and genetic information that it contains. On the sex chromosomes there are genes that are responsible for sex determination and sexual differentiation. The Y chromosome is male specific, but the X chromosome carries many genes involved in basic cellular housekeeping functions or developmental pathways. Females with two X chromosomes have twice the number of these genes; males with a single X have only one dose. Yet, the level of the products of most of these genes is the same in the two sexes.

In the early 1930s, this paradox was first noticed in Drosophila by H.J. Muller while he was studying the eye pigment level of individuals carrying partial loss-of-function X-linked mutations (Muller 1932). Muller reasoned that there must be a regulatory mechanism that helps flies to compensate for the difference in dosage of X-linked genes in males and females by equalizing the level of X-linked gene products between the two sexes. He called this hypothetical regulatory mechanism “dosage compensation”(Fig. 1).

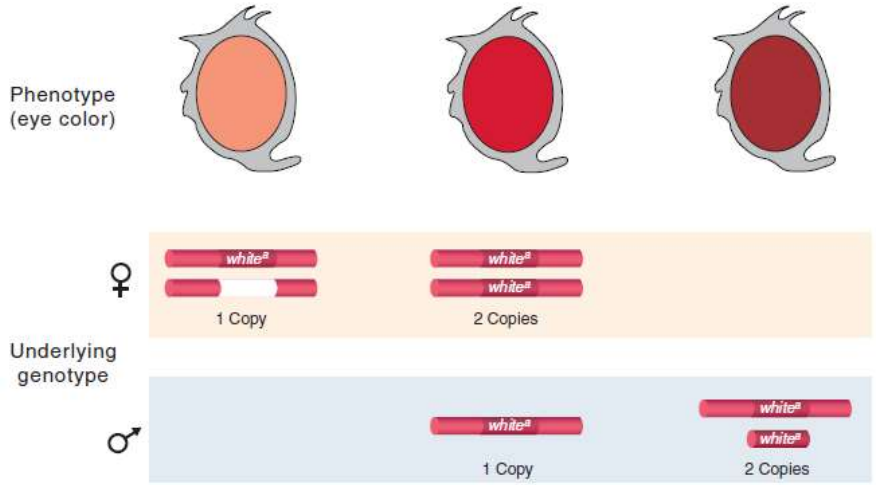


Figure 1. Diagrammatic representation of the results that led H.J. Muller to formulate the hypothesis of dosage compensation. The mutant allele of the X-linked *white* gene (w^a) is a hypomorph and allows partial eye-pigment synthesis; its presence on the X chromosomes is indicated. The level of pigmentation is directly proportional to the dosage of the w^a allele within each sex; yet, males with one dose and females with two doses have comparable amounts of pigment because of dosage compensation.

Following its discovery in Drosophila, the phenomenon of dosage compensation was observed in additional species. In organisms belonging to distantly related groups—from round worms to mammals—transcriptional regulation leading to equal products of X-linked genes in males and females has been achieved in different ways: by decreasing the level of transcription of the two doses of X-linked genes in hermaphrodites relative to males (Caenorhabditis elegans) or by hypertranscribing the X chromosome in both males and females and then shutting down one of the two X chromosomes throughout most of its length in the somatic cells of females (mammals). The mechanisms underlying dosage compensation in these forms are described in Strome et al. (2014) and Brockdorff and Turner (2014).

The first evidence that dosage compensation in Drosophila is achieved by regulating the transcription of X-linked genes was obtained more than 30 years after Muller's seminal observations, by A.S. Mukherjee and W. Beermann (Mukherjee and Beermann 1965). Using transcription autoradiography of the giant polytenic chromosomes of larval salivary glands, a molecular technique that represented the state of the art at that time, these investigators observed that the level of [3H]uridine incorporation by the single X in males and both Xs in females was equivalent. It appeared, therefore, that the rate of RNA synthesis by the single X chromosome in males was approximately twice the rate of each of the two Xs in females. The next experimental breakthrough consisted of the genetic identification by J. Belote and J. Lucchesi of four genes: msl1, msl2, msl3, and mle, with loss-of-function mutations that appeared inconsequential in females but lethal in males; notably, the mutant males showed approximately half of the normal level of [3H]uridine incorporation by their X chromosome (Belote and Lucchesi 1980a,b). Furthermore, the X chromosome had lost its normal paler and somewhat puffed appearance that had been interpreted as an indication of an enhanced level of transcriptional activity in relation to each of the two X chromosomes in females. These results suggested that the equalization of X-linked gene products was achieved by doubling, on average, the transcriptional activity of the X chromosome in males rather than by halving the transcriptional activity of each X in females.

An alternate hypothesis was proposed based on an "inverse dosage effect," in which the activity of all chromosomes is set by general transcriptional regulators (reviewed in Birchler et al. 2011). In males, because of the absence of one X chromosome, a greater concentration of these regulators would be available than in females, driving the activity of all chromosomes to higher levels. For appropriate compensation to occur, the products of the msl loci would sequester some of these regulators away from the autosomes in males, thus leaving only the X chromosome with increased expression. In this model, msl gene mutations result in elevation of the expression of autosomal genes rather than a reduction of X-linked gene expression.

However, a number of experimental results are incompatible with the inverse hypothesis (Arkhipova et al. 1997; Hamada et al. 2005; Straub et al. 2005; Deng et al. 2011). Particularly compelling is the recent observation that ectopic MSL complex on autosomes leads to a localized increase in transcription and suppression of phenotypes caused by haplo-insufficient mutants in the same region (Park et al. 2010).

Among the four genes introduced above, two were newly discovered (male-specific lethal 1, msl1; and male-specific lethal 2, msl2), whereas the other two (maleless, mle; and male-specific lethal 3, msl3) had been previously identified by other investigators in natural populations (specific references to this early phase of the study of dosage compensation can be found in Lucchesi and Manning 1987). For ease of reference, all of the gene products identified to date, on the basis of the male-specific lethal phenotype of their loss-of-function mutations, are called the MSLs. The next phase in the study of dosage compensation was initiated with the cloning of mle and the three msl genes, and the discovery and cloning of the mof histone acetyltransferase gene. By cytoimmunofluorescence, the five gene products were found to associate in an identical pattern at numerous sites along the polytene X chromosome in males (reviewed in Gelbart and Kuroda 2009). This observation and the interdependence of the different gene products for X-chromosome binding suggested that they form a complex. It is crucial for viability that the complex is present in males (XY) and absent in females (XX); therefore, the first step in dosage compensation is to establish this sex specificity.

REGULATORS OF DOSAGE COMPENSATION:

Regulation of Dosage Compensation Starts with Counting the Number of X Chromosomes:

Each embryo needs to count its X chromosomes to make the critical decision whether or not to implement dosage compensation. An incorrect decision, such as failure to upregulate the single male X or aberrant up-regulation of both female XXs, results in lethality. In Drosophila, the X-counting process is coordinated with the sex determination decision. Phenotypic sex is determined by the number of X chromosomes per nucleus, such that XX embryos are females and XY embryos are male. The Y chromosome is required for male fertility, but unlike in mammals, it plays no role in phenotypic sex. Formally it is the X:autosome ratio that controls both sex and dosage compensation, as the X counting mechanism is sensitive to the number of sets of autosomes. This becomes apparent in 2X:3A triploids, which have an intermediate X:A ratio between XY:2A males and XX:2A females. 2X:3A triploids differentiate as intersexes with a mixture of both male and female cells.

The X:A ratio controls both sex determination and dosage compensation by regulating a critical binary switch gene, Sex lethal (Sxl). Sxl encodes a female-specific RNA binding protein that regulates splicing and translation of key messenger RNAs (mRNAs) in the sex determination and dosage compensation pathways respectively (Fig. 2). The Sex lethal gene resides on the X chromosome and is positively regulated by transcription factors encoded by the X, such that embryos with two X chromosomes are able to initiate Sxl expression from an early, regulated promoter, Pe, whereas embryos with a single X per nucleus fail to express Sxl from Pe. This initial transient difference in activation of Sxl in early embryos is stabilized by an autoregulatory loop in which SXL protein positively regulates splicing of its own mRNA from a maintenance promoter that is expressed constitutively. SXL initiates differentiation n the female mode by regulating the splicing of the transformer (tra) gene in a sex-specific manner. In turn, this gene product (together with the product of another gene, transformer2 (tra2), present in both sexes) directs the splicing of the doublesex (dsx) primary transcript to yield a regulatory protein that acts to repress genes required for male development, thus achieving female sexual differentiation. In male embryos, an alternate mode of splicing of the dsx transcripts occurs by default and leads to a product that represses genes required for female development, resulting in male sexual differentiation.

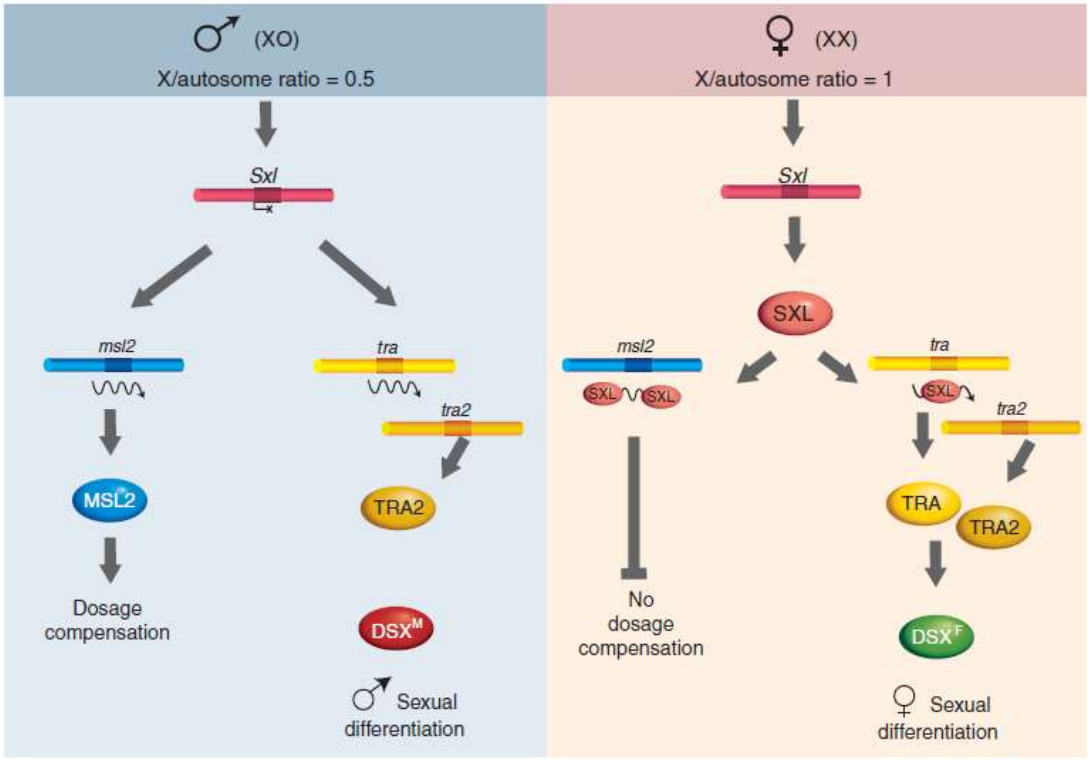


Figure 2. Diagram of the control of sex determination and dosage compensation. If the X/A ratio is equal to 1, a regulatory cascade leads to female sexual development. In females, the presence of the *Sxl* gene product prevents the translation of the *msl2* message and the assembly of the MSL complex. If the X/A ratio is only 0.5, absence of the cascade leads by default to male sexual development and to the formation of the MSL complex.

The SXL Protein Prevents Formation of the MSL Complex in Females:

The key target of SXL in the dosage compensation pathway is msl2 mRNA (Bashaw and Baker 1997; Kelley et al. 1997). SXL binding sites are located in both the 5′ and 3′ untranslated

regions (UTRs) of msl2 mRNA. SXL is normally present only in females, in which it represses translation of the msl2 mRNA through association with its UTRs (see Fig. 2). If SXL is absent in females, dosage compensation is aberrantly turned on and these females die. Conversely, if SXL is ectopically expressed in males, dosage compensation is turned off and males die. Ectopic expression of MSL2 in females is sufficient to assemble MSL complexes on both female X chromosomes, indicating that all other MSL components are either turned on or stabilized by expression of MSL2.

In summary, dosage compensation must respond to the number of X chromosomes in the nucleus, and these are counted early in embryonic development. Females repress MSL2 translation, preventing inappropriate dosage compensation when two X chromosomes are present. In the absence of SXL-mediated repression, males express MSL2 protein and this leads to the assembly of a functional MSL complex.

CHROMATIN MODIFICATIONS ASSOCIATED WITH DOSAGE COMPENSATION:

A key modification that is correlated with the association of the MSL complex with the X chromosome in males is the presence of a high level of histone H4 acetylated at lysine 16 (Turner et al. 1992; Bone et al. 1994). This chromatin mark occurs throughout active transcriptional units with a bias toward the middle and the 3′ end (Fig. 7) (Kind et al. 2008; Gelbart et al. 2009).

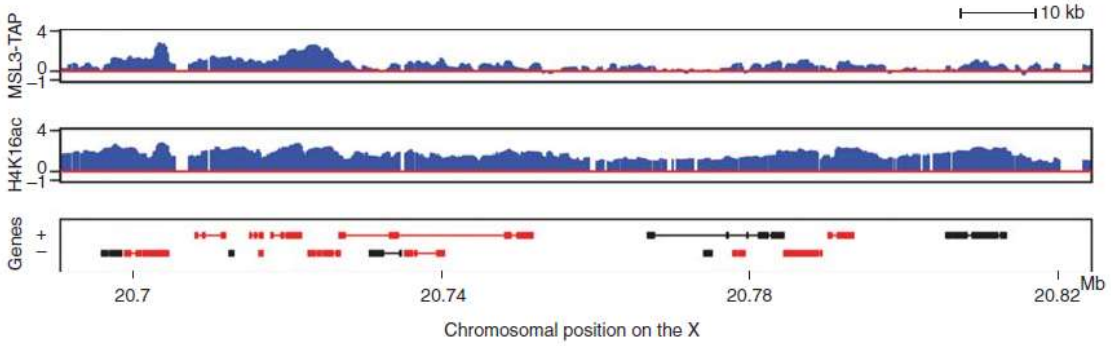


Figure 7. Correlation of H4K16 acetylation and MSL complex binding on the male X chromosomes. The distribution of H4K16ac on the male X chromosome is broader than MSL complex; active genes that lack stable MSL binding are nonetheless associated with H4K16ac. See Figure 5 for explanation of gene representation. (Adapted from Gelbart et al. 2009.)

In yeast, this particular covalent modification of histone H4 plays a key role in maintaining the boundary between silent and active chromatin; loss of function of Sas2, the HAT responsible for H4K16ac, allows the spreading of telomeric heterochromatin into adjacent subtelomeric chromatin (Suka et al. 2002). Structural studies have indicated that a key internucleosomal interaction may occur between an acidic patch of the histone H2A-H2B dimer on one nucleosome and a positively charged segment of the histone H4 tail (residues 16–26) extending from a neighbouring nucleosome (Luger et al. 1997; Schalch et al. 2005). When lysine 16 is acetylated, its positive charge becomes neutral suggesting that weakening a repressive internucleosomal structure could play a key role in dosage compensation. This

contention was supported by the demonstration that reconstituted nucleosomal arrays acetylated at lysine 16 of histone H4 cannot achieve the level of salt-induced condensation of nonacetylated arrays (Shogren-Knaak et al. 2006; Robinson et al. 2008) and that this acetylation also weakens the self-association of reconstituted single nucleosome particles, reflecting the specific role of H4K16 in nucleosome–nucleosome stacking (Liu et al.2011). Using molecular force spectroscopy, the acetylation of H4K16 was observed to weaken nucleosome packing in reconstituted chromatin fibers and to result in a more disordered architecture (Dunlap et al. 2012). Whether, in vivo, it is the formation of the 30-nm fiber (intramolecular compaction) or the higher-order 100- to 400-nm fibers (intermolecular compaction) that are affected by the presence of H4K16ac is not known (Shogren-Knaak et al. 2006). In either case, the presence of H4K16ac renders the chromatin of dosage compensated genes more accessible to factors or complexes. This is evidenced by the significantly greater accessibility of the compensated male X chromosome to an extrinsic DNA-binding protein (e.g., the bacterial DNA methyltransferase). The elevated accessibility
of this protein follows the distribution of H4K16ac along the X (Bell et al. 2010). Given the long-standing correlation between active chromatin and early DNA replication (Hiratani and Gilbert 2009; Schubeler et al. 2002), it is not surprising that another feature of the compensated X chromosome is that it initiates replication earlier in S phase than the rest of the genome (Lakhotia and Mukherjee 1970; Bell et al. 2010).

Another finding is that the X chromosome is more susceptible to mechanical shearing than the autosomes in both males and females, indicating that it has a more open chromatin structure. In fact, the histone marks associated with active gene transcription (H3K4me2 and H3S10ph), as well as those specifically enriched on the dosage compensated X (H4K16ac) in males, are also slightly enriched on the X chromosomes of females. These findings suggest that the evolution of the distinct chromatin structure responsible for dosage compensation in males has affected the female X (Zhang and Oliver 2010).

In addition to the effect of H4K16 acetylation, there is mounting evidence that dosage compensation involves changes in the torsional stress of X-linked genes. Reducing the level of supercoiling factor, a protein known to associate at the 5′ end of active genes (Ogasawara et al. 2007), preferentially affects male viability because of a sex-specific decrease in the transcription of X-linked genes (Furuhashi et al. 2006). Compensated chromatin is topologically different from noncompensated chromatin. The difference requires the function of topoisomerase II, which associates with the MSL complex and is recruited to compensated genes in excess of the amount present on autosomal genes with similar transcription levels (Cugusi et al. 2013). The rate of histone variant H3.3 incorporation into the X chromosome in male cells is enhanced in relation to the autosomes (Mito et al. 2005). This is to be expected, as the replication-independent nucleosome deposition of H3.3 occurs in transcriptionally active regions of chromatin and involves the replacement of histone H3with the variant H3.3 (see Henikoff and Smith 2014). However, contrary to the expectation that this increased level of H3.3 on the X may contribute to the mechanism of dosage compensation is the observation that the absence of the two genes that encode H3.3, although causing sterility in both sexes, has no effect on viability of mutant flies (Hodl and Basler 2009).

DOSAGE COMPENSATION AND NUCLEAR ORGANIZATION:

During interphase, chromosomes are seen to occupy individual territories rather than an intermingling of unravelled chromatin strands (Cremer and Cremer 2010; see Dekker and Misteli 2014). This organization is particularly evident in cells of Drosophila males in which the X chromosome can be identified by the presence of the MSL complex (Strukov et al. 2011). Within this compartment, the chromatin modifications that underlie the mechanism of dosage compensation appear to induce a particular higher order topography to the X chromosome. Throughout development, X-linked sites that are separated by approximately a dozen megabases are located much closer in male than in female nuclei (Fig. 10) (Grimaud and Becker 2009). This difference is dependent on the presence of the MSL1- MSL2 chromatin-recognition component of the complex and is not affected by the absence of the other three MSL proteins. Because, in the absence of any one of the latter, the partial complex that includes MSL1-MSL2 is found only at HASs, the proximity of X-linked loci in male cells must be mediated by their clustering (Grimaud and Becker 2009). It is interesting to note that the MSL proteins copurify with the nuclear pore complex proteins Nup153 and Megator (Mtor). Regions of the genome at the nuclear periphery that are proximal to the nuclear pore complex contain groups of active genes, suggesting this compartment may have a regulatory effect on transcription (Vaquerizas et al. 2010). Depletion of the Nup153 and Mtor nucleoporins leads to the loss of dosage compensation (Mendjan et al. 2006), although this analysis could be complicated by general viability issues (Grimaud and Becker 2009).

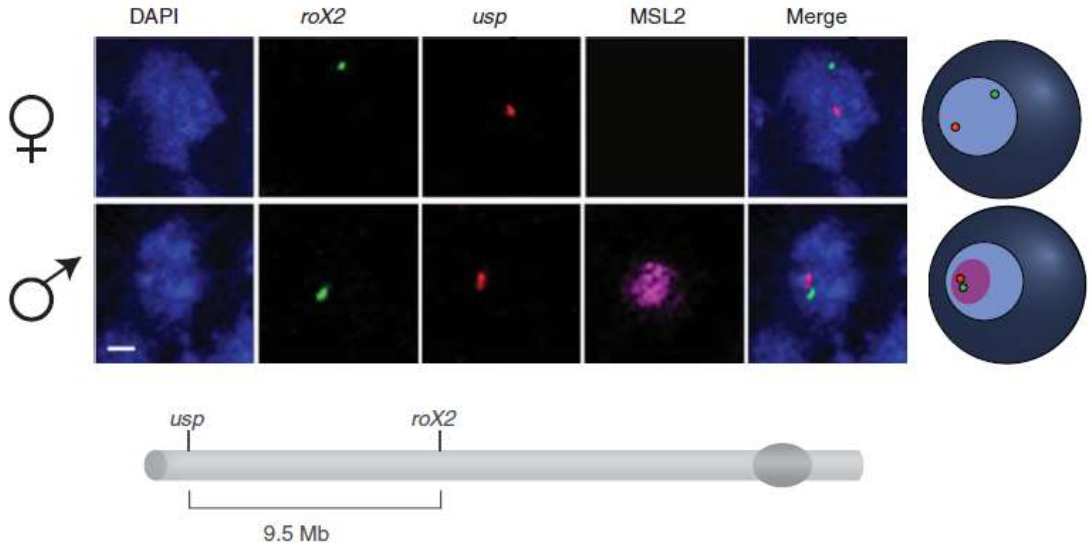


Figure 10. Male-specific conformation of the dosage-compensated X chromosome. A pair of high-affinity chromosomal sites (*roX2* and *usp*) were visualized by two-color FISH (fluorescence in situ hybridization) in female or male embryos. DNA was stained with DAPI (blue) and the X-chromosome territory (magenta) was painted with an antibody against MSL2 in male nuclei (there is no MSL2 in female nuclei). A merge of the channels reveals the proximity of the HASs and their residence relative to the MSL2 territory in male nuclei, clearly summarized in the cartoon on the right. The schematic diagram showing part of the X chromosome below indicates the distances separating the different HASs. (Modified, with permission, from Grimaud and Becker 2009, © Cold Spring Harbor Laboratory Press.)

Dosage Compensation in Human:

Dosage Compensation and Sex-Chromatin Bodies:

In man it has been found that Y-chromosomes are genetically inert in comparison to the X-chromosomes and other chromosomes and only a few genes are present in the human Y-chromosome. From the above discussion on the chromosome numbers of male and female human, it appears that females contain a higher dose of functional gene containing chromosome than males (Female chromosome numbers = 44 + XX and Male chromosome number = 44 + XY).

For many years, geneticists have observed that in some case, female homozygous for the genes in the X-chromosomes do not express a trait more markedly than do hemizygous males. So, it must be a mechanism of "dosage compensation", through which the effective dosage of genes of the two sexes is made equal or nearly so.

This mechanism of compensating the differential doses of functional sex chromosomes in male and female human is effected by the inactivation of one X-chromosome in the normal female. The genetically inactive X- chromosome or condensed X-chromosome is called hetero-pychnotic X-chromosome or heterochromatin or sex-chromatin body or Barr body (according to the name of the geneticist M. L. Barr who first observed it) or Drum-stick (according to the shape of the inactive X-chromosome). Of the two X-chromosomes in females, which X-chromosome becomes inactive is a matter of chance, but it should be remembered that once an X- chromosome has become inactivated, all cells arising from that cell will keep the same inactive X-chromosome. In humans, inactive form of X-chromosome as a Barr-body have been observed by the sixteenth day of gestation. X-chromosome inactivation occurs in human when two or more X-chromosomes are present.

Details about Dosage Compensation or Lyon's Hypothesis:

The inactive X hypothesis or the Lyon's hypothesis or the Dosage Compensation is widely known from 1961 which states that only one of the two X chromosomes in the homogametic sex is functional while the other condenses and is inactivated. The X inactivated in some cells would be that from the father, in other cells it would be that from the mother.

Hence any tissue in the body of a woman would be a mosaic of cells which would show dominance of all genes having diffusible products but would remain a fine-grained mosaic for other intracellular differences.

Such a mosaic of cells might be difficult to demonstrate, particularly among rigid tissues, although cells which can be separated and cloned might show antigenic differences. This hypothesis has stimulated many new investigations, some of which are currently being completed.

Objectives behind the Proposition of Lyon's Hypothesis:

Lyon was impressed by three observations relating to X chromosome:
1. In normal mammalian females, one of the two X's is genetically inactive in the somatic cells (single active X-hypothesis).
2. Inactivation is random i.e., irrespective of paternal and maternal origin (random inactivation).
3. (a) The inactivation occurs during early ontogeny (early ontogenic differentiation) and (b) The particular X which has thus become inactivated, remains inactive in all the succeeding cell generation (fixed differentiation).

Evidences in Support of Lyon's Hypothesis:

A. For Single Active X Hypothesis:
1. Bengham (1958) and Russell (1961) noticed that an XO mouse is normal and fertile female indicating that the activity of the single X is sufficient for the normal development of this species.

2. McNeil (1956) recorded the case of "calico-cat" or tortoise-shell cat, usually a heterogygote female with black and yellow patches. Here the dominant X linked coat- colour gene producing yellow-colour becomes inactivated in some cells, whereas in others this mutant gene produces yellow-colour, thus causing a mosaic appearance. Exceptional male "calico" is XXY.:

3. X-linked ocular albinism in female heterogygotes causes the mosaic pigmentation of retina showing one active and another inactive X.

4. The late replicating nature of sex chromatin by H_3-TdR and very little or no RNA synthesized by Barr body in human body indicate the metabolic activity of one X chromosome.

5. The DNA replication pattern in mammalian females, for example:
Taylor (1960) — in Chinese haunter,
German (1962) — in human being
Mukherjee & Sinha (1966) — in cow etc. shows a late-replicating X (or inactivated) chromosome.

6. In individuals having XXXY or XXX polysomic conditions:
i) There are 2 late Xs.
ii) 2 sex chromatins and
iii) an apparent inactivity of G6PD genes in all but one X chromosome.

From above discussions it is clear that:

$$\left[\begin{matrix}\text{No. of}\\ \text{Barr Body}\end{matrix}\right] = \left[\begin{matrix}\text{No. of}\\ \text{late X's}\end{matrix}\right] = \left[\begin{matrix}\text{No. of}\\ \text{Inactive X}\end{matrix}\right] = \left[\begin{matrix}\text{Total No. of}\\ \text{'X's—1}\\ \text{(in diploid)}\end{matrix}\right]$$

For polyploid cells, Harnden gave a formula as :

1. $\text{No. of sex chromatin} = \text{No. of X} \frac{\text{Polyploidy}}{2}$
2. $(\# X - \frac{P}{2})$

B. For Random Inactivation:

1. Ohno and Catlanah (1962) examined that prophase skin cells of "variegated" mouse where dominant autosomal (eighth chromosome) coat colour gene had been translocated to an X chromosome as used by Russell. In light coloured area the sex chromatin size (obviously the translocated X) was larger than that of sex chromatin in darker patches.

2. Ohno (1963) worked on chinchilla mouse in which the dominant autosomal gene for chinchilla had been translocated onto an X chromosome. In the heterozygote female, the same size variation was observed.

3. Very distinct and ingenious first autoradiographic evidence for random inactivation was provided by Mukherjee and Sinha (1964) who had labelled in vitro the mule (hybrid of female horse + male donkey) leucocytes with H_3 thymidine at the terminal part of the "S"-period. In about 50% cells, the metacentric horse X was late replicating while submetacentric donkey — Xs were so in the remaining cells. Similar observations had been made in several hybrids such as "Zepony" (male zebra + female pony). "Gazel", "Nikosia" etc.

It has conclusively been noticed that any structurally abnormal X, e.g., ring-X, iso-X, deleted-X etc. is consistently late replicating and, as such, is heavily labelled in autoradiographic experiments. Random inactivation of either the maternal X or the paternal X chromosome seems to be the rule and the continued typical functioning of the single X-chromosome seems to take place early in development. Selection during embryonic growth may favour those cells which retains the normal X on active status. However, the early decision apparently is final for the inactivation of the one of the two X chromosomes.

C. For Early Embryonic Differentiation:

1. Graham (1954) while surveying the sex chromatin, a various species of mammalian females showed the absence of this structure in early embryos.

2. Despite its absence during early ontogeny, Barr bodies are noticed in late embryos of female cat.

3. Sex chromatins are deleted not earlier than five days in rabbit blastocyst and sixteen and nineteen days in human and macaque embryos, respectively.

4. Hills and Yunis (1966, 68) studied the golden hamster embryos from two-celled stage to about five to six days with H_3-TdR and found that late replicating X in female embryos is absent up to eight-celled stage and did identify the late X along the time of implantation at about the fourth day of gestation.

D. For Fixed Differentiation:

1. G6PD, which is much common in Negros and Mediterranean people but is virtually absent in North American whites in deficient activity, is quantitated by its ability for reducing methaemoglobin with the consequent destruction of glutathione.

2. The enzymatic activity is now very conveniently assessed by the action on the substrate and also the detection is made by starch-gel electrophoresis.

3. By "cloning" (aggregation of cells presumably originating from a single progenitor cell), the cells from females, heterozygotes for G6PD activity and employing mostly electrophoretic technique, two cell populations- one enzymatically active and other deficient— were detected.

Recruitment of the Dosage Compensation Complex to the X chromosome in *C. elegans*

In *C. elegans*, the Dosage Compensation Complex (DCC) binds to and represses both X chromosomes in XX hermaphrodites by an average of two-fold. At the core of the DCC is a specialized condensin complex. Condensins are evolutionarily conserved five-subunit protein complexes that are essential for proper chromosome condensation and segregation. In metazoans, two types of condensin complexes (named I and II) share two Structural Maintenance of Chromosomes (SMC) protein subunits, and a set of three different non-SMC subunits. The condensin core of the DCC shares four out of five subunits with condensin I, but includes an SMC variant called DPY-27.

DPY-27 interacts with at least five other non-condensin proteins including SDC-1, SDC-2, SDC-3, DPY-30 and DPY-21. Sex-specificity of the DCC is provided by SDC-2 protein, which is expressed only in XX hermaphrodites during early embryogenesis. SDC-2, SDC-3 and DPY-30 are required for the recruitment of the condensin portion of the DCC to the X chromosome. *C. elegans* DCC first binds to a number of recruitment sites on the X (*rex*), and then spreads onto the X chromosome. There are approximately 100 predicted *rex* sites along the length of the 17.5 Mb X-chromosome. Initially, *rex* sites were identified by assaying the ability of DNA fragments, in the form of multi-copy extrachromosomal arrays to recruit the DCC. ChIP-chip analysis of DCC identified additional recruitment sites, and defined a 10 bp DNA sequence motif that is enriched at the *rex* sites. This motif was later extended to 12-bp and named the motif enriched on the X (MEX). Although a 35 bp DNA fragment containing the motif as shown to recruit the DCC on extrachromosomal arrays, it is still unknown if the same fragment could recruit as a single copy insertion on an autosome. Nevertheless, the extrachromosomal

recruitment assays show that MEX is important, because mutation of the motif resulted in loss of DCC recruitment. It is not known if any of the DCC subunits bind directly to MEX. Therefore, it remains unclear which proteins specify X-recruitment of the DCC via interaction with the MEX motif.

Mechanism of chromatin remodelling

Nucleosomes inhibit transcription, DNA repair, and other chromosome transactions. SWI/SNF and RSC chromatin-remodelling complexes relieve this inhibition by sliding or disassembling nucleosomes. In the case of sliding, the structure of the nucleosome is unaltered at the end of the reaction. Studies to date have revealed an important principle of the remodelling process, DNA translocation driven by ATP hydrolysis, but the underlying mechanism remains obscure. Translocation slides the DNA around the histone octamer. Sliding exposes DNA at one end of an isolated nucleosome particle and in the linker regions between nucleosomes in an array. Exposure of the DNA may be detected by an increase in susceptibility to attack by nucleases.
Translocation is effected by the Sth1 subunit of RSC, a member of the DEAD/H-box family of helicase/translocases. These enzymes contact DNA through two domains and step along one strand by a scissors-like motion between them. A gap in one strand stops this stepping and blocks translocation. It could be inferred from the effect of gaps in nucleosomal DNA that Sth1 contacts one strand about two turns from the dyad of the nucleosome. A favored notion for remodelling is that the translocase draws DNA into the nucleosome from one side, creating a bulge, which is expelled on the other side. The alternative of DNA twisting strain rather than a bulge traversing the nucleosome has been excluded on the basis of studies with nicked or gapped nucleosomal DNA.
An outstanding question for remodelling by DNA translocation is how the remodeler effects DNA sliding in the face of histone–DNA interaction. Translocation by Sth1 entails DNA sliding from the end of the nucleosome to a point near the dyad, requiring the disruption of all histone–DNA contacts along the way. Neither the mechanism nor the energetics of this process has been described.

Probable questions:

1. Discuss how dosage compensation was discovered in *Drosophila*?
2. How number of X chromosome regulate the dosage compensation in *Drosophila*?
3. How chromatin modification is associated with dosage compensation?
4. how dosage compensation is related with nuclear organization.
5. Discuss Lyon's hypothesis.
6. Discuss some evidences in support of Lyon's hypothesis.
7. How dosage compensation occurs in *C. elegans*?
8. Discuss the mechanism of chromatin remodelling.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

Unit-V

Epigenetics and genome imprinting - DNA methylation in mammals, genomic imprinting in mammals

Objective: In this unit we will discuss about genomic imprinting and DNA methylation in mammals.

Introduction:

The presence of genomic imprinting in mammals has considerable medical, societal, and intellectual implications in terms of (1) the clinical management of genetic traits and diseases, (2) the capacity to control human and animal breeding by assisted reproductive technologies, and (3) the progress of biotechnology and postgenomic medical research. Any modern day discussion of genetic problems, whether in research or medicine, must consider if a gene shows a biparental (i.e., diploid) mode of expression, or, is subject to genomic imprinting and shows parental-specific (i.e., haploid) expression. Despite the importance of genomic imprinting to human health and well-being, it is surprising that widespread acceptance of its existence and significance did not happen until the early nineties after three genes were unequivocally shown to display parental specific expression in mice.

Parental-specific behaviour of whole chromosomes had been observed in cytogenetic studies of chromosomes in Arthropods as early as the 1930s (Chandra and Nanjundiah 1990). Interestingly, the term “chromosome imprinting” was first coined to describe paternal-specific chromosome elimination that plays a role in sex determination in some Arthropod species (Crouse et al. 1971). Chromosomal imprinting of the mammalian X chromosome was also noted, which leads to paternal-specific inactivation of one of the two X chromosomes in all cells of female marsupials and the extraembryonic tissues of the mouse (Cooper et al. 1971). During the same period, classical geneticists were generating mouse mutants carrying chromosomal translocations that laid the foundation for the observation of imprinted gene expression. Some of these “translocation” mice, initially used to map the position of genes on chromosomes, showed a parental-specific phenotype when certain chromosomal regions were inherited as duplications of one parental chromosome in the absence of the other parental chromosome (known as uniparental disomy or UPD; Fig. 1). These results indicated the possibility “that haploid expression of particular maternal or paternal genes is important for normal mouse development” (Searle and Beechey 1978). At the same time, other geneticists used an unusual mouse mutant known as the “hairpin-tail” mouse that carried a large deletion of chromosome 17 to unequivocally set aside a basic tenet of genetics “that organisms heterozygous at a given locus are phenotypically identical irrespective of which gamete contributes which allele to the genotype” (Johnson 1974). Instead, offspring who received the Hairpin-tail deletion from a maternal parent were increased in size and died midway through embryonic development, whereas paternal transmission of the genetically identical chromosome produced viable and fertile mice (Fig. 1). It is notable with hindsight that in spite of the previously published description of imprinted X-chromosome inactivation in mammals,

the favoured interpretation of these genetic translocation and deletion experiments was not that the regions contained imprinted genes, but that genes on these autosomes primarily acted in the haploid egg or sperm to modify proteins used later in embryonic development. Despite this, the concept of differential functioning of the maternal and paternal genome was gaining ground and a suggestion made that "the maternal genome might be normally active at the Hairpin-tail chromosomal region while its paternal counterpart is preferentially inactivated" (McLaren 1979).

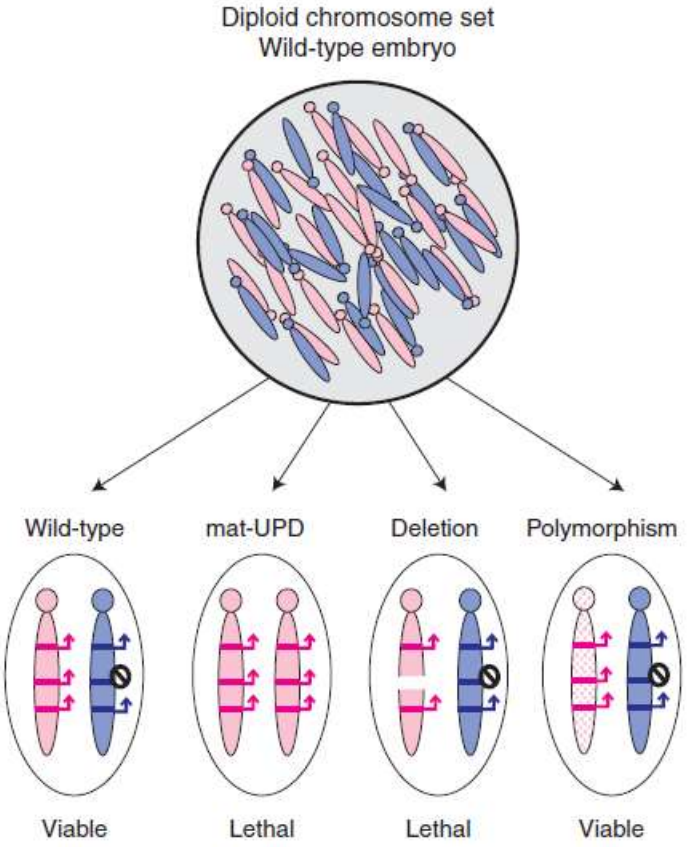


Figure 1. Mouse models to study genomic imprinting that allow the maternal and paternal chromosome to be distinguished. Mammals are diploid and inherit a complete chromosome set from the maternal and paternal parent. However, mice can be generated that (1) inherit two copies of a chromosome pair from one parent and no copy from the other parent (known as UPD), (2) inherit a partial chromosomal deletion from one parent and a wild-type chromosome from the other parent, and (3) inherit chromosomes carrying single-nucleotide polymorphisms (known as SNPs) from one parent and a wild-type chromosome from the other parent. Offspring with UPDs or deletions are likely to display lethal phenotypes, whereas SNPs will allow the production of viable offspring.

A major step forward in establishing the existence of genomic imprinting in mammals came several years later with the development of an improved nuclear transfer technology being used to test the possibility of generating diploid uniparental embryos solely from mouse egg nuclei. The nuclear transfer technique took a donor male or female pronucleus from a newly fertilized egg and used a fine micropipette to place it inside a host fertilized egg from which either the

maternal or paternal pronucleus had been removed. This regenerated diploid embryos, but with two maternal or two paternal genomes (known, respectively, as gynogenetic and androgenetic embryos; Fig. 2). The technique was first used to show that nuclei from fertilized Hairpin-tail mutant embryos could not be rescued when transferred into a wild-type host egg. This provided proof that the embryonic genome, and not the oocyte cytoplasm, carried the Hairpin-tail defect. It also confirmed the suggestion that genes on the maternal and paternal copy of chromosome 17 functioned differently during embryonic development (McGrath and Solter 1984b). Subsequently, nuclear transfer was used to show that embryos, reconstructed from two maternal pronuclei (known as gynogenetic embryos) or two paternal pronuclei (androgenetic embryos), failed to survive; whereas only embryos reconstructed from one maternal and one paternal pronucleus produced viable and fertile offspring (McGrath and Solter 1984a; Surani et al. 1984). This work overturned a previous claim that uniparental mice could develop to adulthood (Hoppe and Illmensee 1982). Gynogenetic embryos at the time of death were defective in extraembryonic tissues that contribute to the placenta, whereas androgenetic embryos were defective in embryonic tissue. These outcomes led to the hypothesis that embryonic development required imprinted genes expressed from the maternal genome, whereas the paternal genome expressed imprinted genes required for extraembryonic development (Barton et al. 1984). Subsequent identification of imprinted genes in the mouse did not confirm a bias in the function of imprinted genes, but indicated that the observed differences between gynogenetic and androgenetic embryos may be explained by a dominant effect of one or a few imprinted genes. The nuclear transfer experiments, combined with supporting data from mouse genetics, provided convincing evidence that both parental genomes were required for embryogenesis in mice, laying a strong foundation for the existence of genomic imprinting in mammals (Fig. 2). An extensive survey of parental chromosome contribution to embryonic development, using “translocation” mice to create UPD chromosomes (Fig. 1), identified two regions on mouse chromosomes 2 and 11 that showed opposite phenotypes when present either as two maternal or two paternal copies.

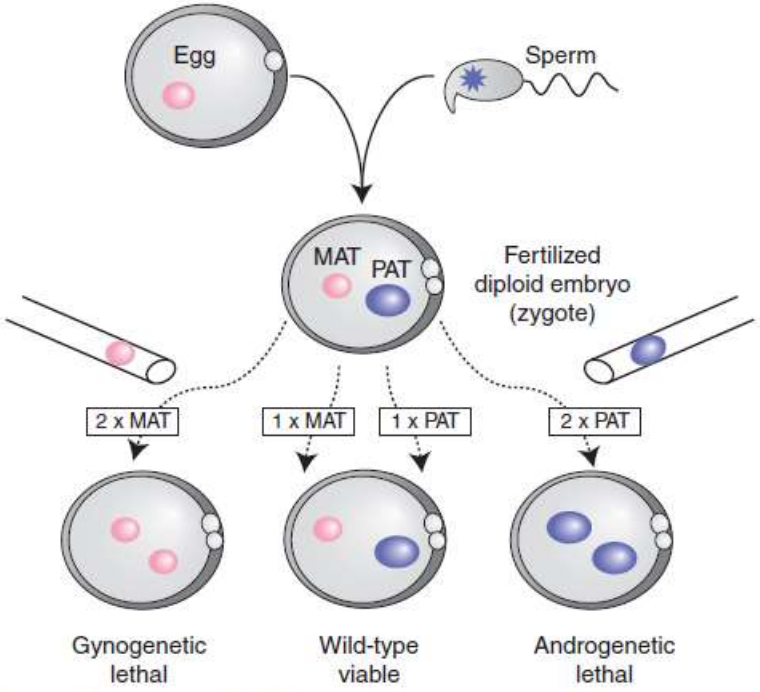


Figure 2. A maternal and paternal genome are needed for mammalian reproduction. The nuclear transfer technique used micropipettes and high-powered microscopes to remove the male or female nuclei from a newly fertilized egg and place them in various combinations into a second "host" fertilized egg that had already been enucleated, thereby generating anew diploid embryos with two maternal (gynogenetic) or two paternal (androgenetic) genomes or a biparental genome (wild-type). Gynogenetic and androgenetic embryos were lethal at early embryonic stages. Only reconstituted embryos that received both a maternal and paternal nucleus (wild-type) survived to produce living young. These experiments show the necessity for both the maternal and paternal genome in mammalian reproduction, and indicate the two parental genomes express different sets of genes needed for complete embryonic development.

This further strengthened the argument for parental- specific gene expression in mammals (Cattanach and Kirk 1985). In addition, human data strongly indicated that some genetic conditions, most notably the Prader– Willi syndrome, which appears to arise exclusively by paternal transmission, could best be explained by parental-specific gene expression (Reik 1989). Further clues came from experiments applying the newly developed technology for making transgenic mice by microinjecting gene sequences into a fertilized mouse egg. This was often beset by the problem of DNA methylation unexpectedly inducing silencing of the transgene in somatic tissues. Some transgenes even showed parental-specific differences in their ability to acquire DNA methylation, adding weight to the argument that parental chromosomes behave differently. This normally followed the pattern that maternally transmitted transgenes were methylated whereas paternally transmitted transgenes were not. However, only in a few cases did DNA methylation differences correlate with parental-specific expression. Although many similarities were later found between "transgene" methylation

imprinting and genomic imprinting of endogenous mouse genes, several features distinguish them (Reik et al. 1990). This includes a high susceptibility to strain-specific background effects, an inability to maintain imprinted expression at different chromosomal integration sites, and a requirement for foreign DNA sequences to produce the imprinted effect (Chaillet et al. 1995).

Despite the wealth of supportive data, final proof of the existence of genomic imprinting in mammals depended on the identification of genes showing imprinted parental specific expression. This occurred in 1991 when three imprinted mouse genes were described. The first of these, Igf2r (insulin-like growth factor type 2 receptor that is a "scavenger" receptor for the growth hormone insulin-like growth factor type 2 [Igf2]) was identified as a maternally expressed imprinted gene. This gene was later shown to explain the overgrowth phenotype of the Hairpin-tail mutant mouse (Barlow et al. 1991). A few months later, the Igf2 gene was identified as a paternally expressed imprinted gene (DeChiara et al. 1991; Ferguson-Smith et al. 1991). Finally, the H19 gene (cDNA clone number 19 isolated from a foetal hepatic library), an unusual long noncoding RNA (lncRNA) was subsequently shown to be a maternally expressed imprinted gene (Bartolomei et al. 1991). Diverse strategies were used to identify these three imprinted genes, each of which depended on emerging technologies in mouse genetics. For Igf2r, positional cloning was used to identify genes that mapped to the Hairpin-tail deletion on chromosome 17. Mice then inheriting the deletion from one parent were used to identify those genes showing maternal-specific expression (Fig. 1). For Igf2, the physiological role of this growth factor in embryonic development was being tested by gene knockout technology.

Surprisingly, mice carrying the mutant non-functional allele showed a phenotype following paternal transmission, but no phenotype on maternal transmission. The H19 lncRNA was identified as an imprinted gene after this gene was mapped close to the Igf2 locus on chromosome 7, proving the hypothesis that imprinted genes could be clustered together. Although these strategies were to prove useful in subsequent attempts to identify imprinted genes, the demonstration that imprinted genes were closely clustered has proven to be a pivotal discovery in understanding the mechanism controlling genomic imprinting in mammals.

Genomic Imprinting—An Epigenetic Gene Regulatory System:

The defining characteristic of genomic imprinting is that it is cis acting. Thus, the imprinting mechanism acts only on one chromosome. The two parental chromosomes will normally contain many single base pair differences (known as single-nucleotide polymorphisms [SNPs]) if the population is outbred, but they can be genetically identical if inbred mouse strains are used. Because genomic imprinting occurs in inbred mice that have genetically identical parental chromosomes, it was concluded that the process must use an epigenetic mechanism to modify the information carried by the DNA sequence, yet create an expression difference between the two parental gene copies. These observations also indicate that a cis-acting silencing mechanism, which is restricted to one chromosome, is operating so that the silencing factors cannot freely diffuse through the nucleus to reach the active gene copy. Although imprinted genes are repressed on one parental chromosome relative to the other, genomic imprinting is not necessarily a silencing mechanism and has the potential to operate at any level

of gene regulation (i.e., at the promoter, enhancers, splicing junctions, or polyadenylation sites) to induce parental-specific differences in expression. Genomic imprinting must therefore depend on an epigenetic system that modifies or "imprints" one of the two parental chromosomes (Fig. 3). This imprint is subsequently used to attract or repel transcriptional factors or mRNA processing factors, thereby changing expression of the imprinted gene on one parental chromosome. Because inbred mice with genetically identical chromosomes also show genomic imprinting, parental imprints are not likely to be acquired after the embryo becomes diploid because there would be no way for the cells' epigenetic machinery to distinguish between identical parental gene copies. Thus, parental imprints must be acquired when the two parental chromosome sets are separate and this only occurs during gamete formation and for 12h postfertilization (Fig-3).

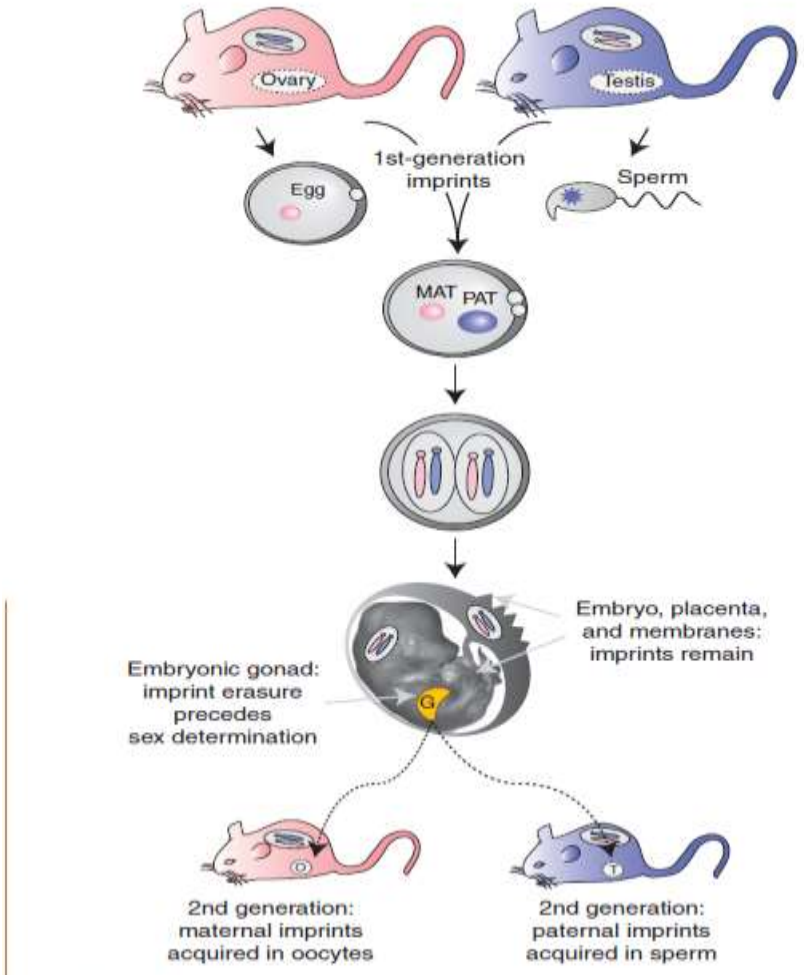


Figure 3. Imprint acquisition and erasure in mammalian development. Imprints are acquired by the gametes; thus, oocytes and sperm already carry imprinted chromosomes (first-generation imprints). After fertilization when the embryo is diploid, the imprint is maintained on the same parental chromosome after each cell division in cells of the embryo, yolk sac, placenta, and also in the adult. The germ cells are formed in the embryonic gonad and the imprints are erased only in these cells before sex determination. As the embryo develops into a male, the gonads differentiate to testes that produce haploid sperm that acquire a paternal imprint on their chromosomes. Similarly, in developing females, chromosomes in the ovaries acquire maternal imprints (second-generation imprints).

The most likely scenario is that gametic imprints are placed on paternally imprinted genes during sperm production and on maternally imprinted genes during egg formation.
A key feature of the "imprinted" DNA sequence is that it would only be modified in one of the two parental gametes; thus, two types of recognition system are required, one sperm-specific and one oocyte-specific, each directed toward a different DNA sequence. Several other features are required of the imprint. First, once established, it must remain on the same parental chromosome after fertilization when the embryo is diploid. Second, the imprint must be stably inherited through mitosis of the embryo and adult animal. Last, it must be erasable. The latter is necessary because the embryo will follow either a male or female developmental path midway through development and its gonads will need to produce only one type of imprinted haploid parental gamete. Thus, germ cells that have arisen from embryonic diploid cells (Fig. 3) must first lose their inherited maternal and paternal imprints before they gain that of the gamete.
An imprint can be defined as the epigenetic modification that distinguishes the two parental copies of a given gene. Once formed, the imprint must also allow the transcription machinery to treat the maternal and paternal gene copy differently within the same nucleus. A gametic imprint is predicted to be continuously present at all developmental stages (Fig. 3), thus imprints can be found by comparing epigenetic modifications on maternal and paternal chromosomes in embryonic or adult tissues (using strategies outlined in Fig. 1) and tracing them back in development to one of the two gametes. Candidates for gametic imprints could be modifications of DNA or histone proteins that package DNA into chromosomes (Allis et al. 2014). There are now two types of epigenetic DNA modification known in mammals; 5-methylcytosine and 5-hydroxymethylcytosine (Li and Zhang 2014). Histones can bear multiple types of modification including methylation, acetylation, phosphorylation, sumoylation, and ubiquitylation (Allis et al. 2014). They can also be replaced by variant histones with specific functions (Henikoff and Smith 2014). Any of these epigenetic modifications could qualify as an imprint. One would predict that enzymes responsible for these epigenetic modifications or an essential cofactor would be exclusively expressed in one of the two gametes, and specifically associate with one parental chromosome to copy the modification when the cell divides. However, as will be described in Section 3 on "key discoveries," only 5-methylcytosine has been clearly shown to function as the gametic imprint for imprinted genes in mammals and, to date, is the only known heritable modification.

How does a gametic imprint control imprinted expression?

To understand how the imprint operates, three pieces of information are required: which parental chromosome carries the imprint, which parental chromosome carries the expressed allele of the imprinted gene, and the position of the imprinted sequence relative to the expressed or silenced allele of the imprinted gene. Using this type of approach it has been shown that gametic imprints can act on whole clusters of genes at once. These imprinted clusters contain 3–12 imprinted genes and span from 100–3700 kb of genomic DNA. The majority of genes in any one cluster are imprinted protein-coding mRNA genes; however, at least one is always an imprinted lncRNA.
Because of the arrangement of imprinted genes in clusters, with some genes expressed from one parental chromosome and some from the other, it is not trivial to determine how the imprint

operates. It is possible to study the effect of the imprint on single genes in the cluster, but it may prove more informative to study the effects of the imprint on the entire cluster. One thing, however, is clear. Nature has not chosen the simplest mechanism whereby the imprint is directed toward a promoter to pre-emptively silence an imprinted gene in one gamete. Instead, imprints appear, in general, to be directed toward long-range cis-acting regulators that influence the expression of multiple genes, and are located a long distance away on the same chromosome.

Imprinted Genes Control Embryonic and Neonatal Growth:

What is the function of genomic imprinting in mammals?

One way to answer this question is to determine the function of known imprinted genes in vivo. This can be performed by mutating the gene sequence to impair its function using the "homologous recombination" technique. The function of many of the known imprinted genes has been determined in this fashion. The most significantly represented function among imprinted genes includes genes that affect growth of the embryo, placenta, and neonate. In this category are paternally expressed imprinted genes that function as growth promoters (i.e., Igf2, Peg1, Peg3, Rasgrf1, Dlk1) and show growth retardation in embryos deficient for the gene. There are also maternally expressed imprinted genes that function as growth repressors (i.e., Igf2r, Gnas, Cdkn1c, H19, Grb10), as shown by a growth enhancement in embryos deficient for the gene. Another significant category includes genes with behavioural or neurological defects (e.g., Nesp, Ube3a, Kcnq1). These results are, at one level, disappointing because they do not identify one function for all imprinted genes. Nevertheless, these results show that the majority of imprinted genes function as embryonic or neonatal growth regulators. More interestingly, the ability to regulate growth appears to be neatly divided with maternally expressed growth regulating genes acting to repress growth of the offspring, whereas paternally expressed genes in this category act to increase growth. Moreover, numerous tested imprinted genes are active in neurological processes, some of which affect neonatal growth rate by altering maternal behaviour.

The Function of Genomic Imprinting in Mammals:

Assessment of genomic imprinting in different types of mammals has been informative. Placental mammals such as mice and humans, and marsupials such as opossum and wallaby, have genomic imprinting. Egg-laying mammals, such as platypus and echidna, appear to lack imprinted genes, although extensive studies have not yet been performed (Renfree et al. 2009). Placental mammals and marsupials are distinguished from egg-laying mammals by a reproductive strategy that allows the embryo to directly influence the amount of maternal resources used for its own growth. In contrast, embryos that develop within eggs are unable to directly influence maternal resources. Most invertebrates and vertebrates use an egg-laying reproductive strategy. Notably, they can also undergo parthenogenesis—a form of reproduction in which the female gamete develops into a new diploid individual without fertilization by a male gamete (note that parthenogenetic embryos arise from the duplication of the same maternal genome, whereas the gynogenetic embryos described in Fig. 2 arise from two

different maternal genomes). The ability of organisms to undergo parthenogenesis most likely indicates a complete absence of genomic imprinting as it shows the paternal genome is dispensable. In mammals, however, a direct consequence of imprinted gene expression controlling foetal growth is that parthenogenesis is not possible. Both parents are necessary to produce viable offspring making mammals completely reliant on sexual reproduction to reproduce (Fig. 4). Parthenogenesis has thus not yet been observed in mammals despite claims to the contrary, although manipulating expression of the Igf2 and Dlk1 imprinted clusters has generated some rare mice with a diploid maternal genome (Kawahara et al. 2007).

Why should genomic imprinting have evolved only in some mammals, but not in vertebrates in general? Three features of genomic imprinting—the growth regulatory function of many imprinted genes, the restriction of imprinted genes to placental and marsupial mammals, and last, the necessity of the paternal genome for foetal development, provide evidence that can fit two equally attractive hypotheses. The first hypothesis proposes that genomic imprinting evolved in response to a "parental conflict" situation. This arises from the opposing interests of the maternal and paternal genome: Embryonic growth is dependent on one parent, but influenced by an embryo whose genome comes from two parents. Paternally expressed imprinted genes are proposed to increase embryonic growth, thereby maximizing the fitness of an individual offspring bearing a particular paternal genome. Maternally expressed imprinted genes are proposed to suppress foetal growth. This would allow a more equal distribution of maternal resources to all offspring and increase transmission of the maternal genome to multiple offspring, which may have different paternal genomes. The second hypothesis is named "trophoblast defence" (Varmuza and Mann 1994). This proposes that the maternal genome is at risk from the consequences of being anatomically equipped for internal reproduction should spontaneous oocyte activation lead to full embryonic development. Because males lack the necessary anatomical equipment for internal reproduction, they do not share the same risks should spontaneous activation of spermatozoa occur. Imprinting is thus proposed to either silence genes on the maternal chromosome that promote placental development or to activate genes that limit this process. The genes necessary for placental invasion of the maternal uterine vasculature would consequently only be expressed from a paternal genome after fertilization has occurred.

Which, if any, of these hypotheses explains the evolution of genomic imprinting in mammals? Both hypotheses indicate a role for imprinted genes in regulating the development and function of the placenta, however, neither the parental conflict nor the trophoblast defence models can provide a full explanation for all the data (Wilkins and Haig 2003). It is interesting to note that imprinted genes have also been identified in the plant endosperm, a tissue that has been compared to the placenta by virtue that it transfers nutrient resources from the parent plant to the embryo (Grossniklaus and Paro 2014). This finding strengthens arguments that genomic imprinting evolved as a means to regulate nutrient transfer between the parent and offspring, but it does not tell us why. Fuller or alternative explanations of the function of genomic imprinting in mammals could come from two sources. The first would be to examine the function of "imprinting" across a complete gene cluster in contrast to examining the phenotype of mice lacking a single imprinted gene product. This would require an ability to reverse an imprint and generate biparental gene expression across the whole imprinted cluster. The second approach is to learn exactly how genes are imprinted. It is possible that not all genes in a cluster

are deliberate targets of the imprinting mechanism and that some may just be "innocent bystanders" of the process, and their function would not be informative about the role of genomic imprinting. The existence of innocent bystander genes affected by the imprinting mechanism may satisfactorily explain the curious abundance of imprinted genes with no obvious biological function in development.

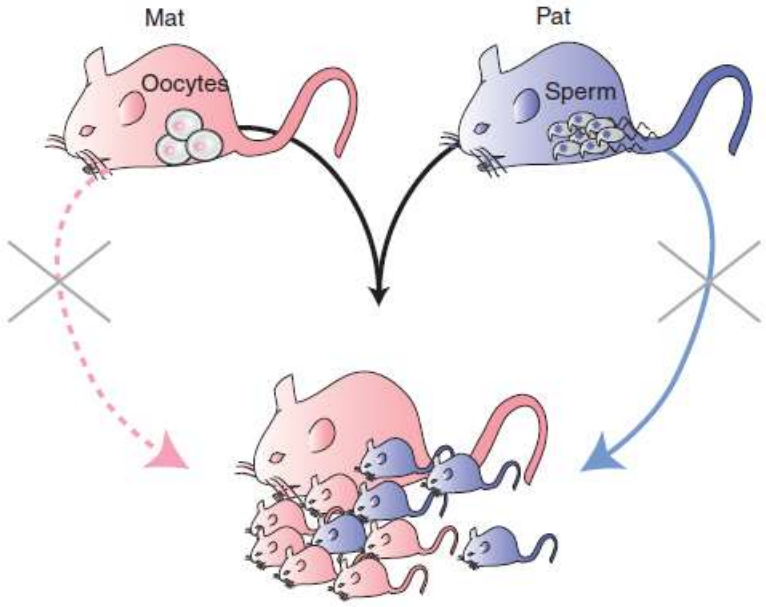


Figure 4. Imprinted genes play a role in mammalian reproduction. Mammals are diploid and reproduction requires fertilization of a haploid female egg by a haploid male sperm to recreate a diploid embryo. Only females are anatomically equipped for reproduction, but they cannot use parthenogenesis to reproduce (the possibility of which is represented by a pink dashed line) because essential imprinted genes needed for fetal growth are imprinted and silenced on maternal chromosomes. These genes are expressed only from paternal chromosomes; thus, both parental genomes are needed for reproduction in mammals. Parthenogenesis is the production of diploid offspring from two copies of the same maternal genome.

Imprinted Genes Are Clustered and Controlled by Imprint Control Elements (ICEs):
To date, about 150 imprinted genes have been mapped to 17 mouse chromosomes including the X chromosome. More than 80% of the identified imprinted genes are clustered into 16 genomic regions that contain two or more genes (Wan and Bartolomei 2008). The discovery of clusters of imprinted genes was a strong indication that a common DNA element may regulate imprinted expression of multiple genes in cis. To date, seven of the 16 imprinted clusters have been well characterized, and these are listed in Table 1 by the name of the principle imprinted mRNA gene in the cluster or after a disease association (e.g., the Pws cluster for Prader–Willi syndrome; Beaudet and Zoghbi 2014). These seven clusters contain three to 12 (or more) imprinted genes and are spread over 80–3700 kb of DNA. A common feature of these seven clusters is the presence of a DNA sequence carrying a gametic methylation imprint that is known as a gametic DMR (differentially DNA-methylated region). A gametic DNA methylation imprint is defined as a methylation imprint established in one

gamete and maintained only on one parental chromosome in diploid cells of the embryo. In five clusters (Igf2r, Kcnq1, Gnas, Grb10, and Pws), the gametic DMR has a maternal methylation imprint acquired in oogenesis, whereas in two clusters (Igf2 and Dlk1), it has a paternal methylation imprint acquired during spermatogenesis. In these examples, the gametic DMR controls imprinted expression of the whole or part of the cluster and is therefore designated as the imprint control element, or ICE, for the cluster (Barlow 2011).

Table 1. Features of imprinted gene clusters in the mouse genome

Cluster name	Chromosome mouse/human	ICE (gametic methylation imprint)	Cluster size (kb)	Gene number in cluster	Parental expression M/P	lncRNA and expression (M or P)
Igf2r	17/6	Region 2 (M)	490	4	3 M (pc) 1 P (nc)	*Airn* (P)
Kcnq1	7/11	*KvDMR1* (M)	780	12	11 M (pc) 1 P (nc)	*Kcnq1ot1* (P)
Pws	7/15	*Snrpn-CGI* (M)	3700	>8	2 M (pc)/ >7 P (nc and pc)	*Ube3aas* (P)[a] *Ipw* (P)[a] *Zfp127as* (P)[a] *PEC2* (P)[a] *PEC3* (P)[a] *Pwcr1* (P)[a]
Gnas	2/20	*Nespas* DMR (M)	80	7	2 M (pc) 5 P (4 nc and 1 pc)	*Nespas* (P)[b] *Exon1A* (P) *miR-296* (P)[b] *miR-298* (P)[b]
Grb10	11/7	Meg1/Grb10 DMR (M)	780	4	2 M (pc)/ 2 P (pc)	NI
Igf2	7/11	H19-DMD (P)	80	3	1 M (nc)/ 2 P (pc)	*H19* (M)
Dlk1	9/14	IG-DMR (P)	830	>5	>1 M (nc)/ 4 P (pc)	*Gtl2* (M)[c] *Rian* (M)[c] *Rtl1as* (M)[c] *Mirg* (M)[c] miRNAs (M)[c] snoRNAs (M)[c]

Note that cluster size and number of genes in the cluster are provisional and await a genome-wide analysis of imprinted expression. Pws and Dlk1 clusters contain overlapping transcripts in which the number of distinct genes is not yet clear. Details are given in the text.

M, maternal; P, paternal; DMR, differentially methylated region; pc, protein coding; nc, noncoding RNA; NI, none identified; miRNA, micro RNA; snoRNA, small nucleolar RNAs.

[a] May be one long lncRNA.

[b] Part of *Nespas* transcript.

[c] May be part of one or multiple lncRNAs.

Table 1 shows that each imprinted gene cluster contains multiple mRNAs and, with the exception of Grb10, at least one lncRNA. Two trends emerge. First, the imprinted protein-coding genes in each cluster are expressed, for the most part, from the same parental chromosome, whereas the lncRNA is expressed from the opposite parental chromosome (as illustrated in Fig. 5 for a maternal gametic DMR).

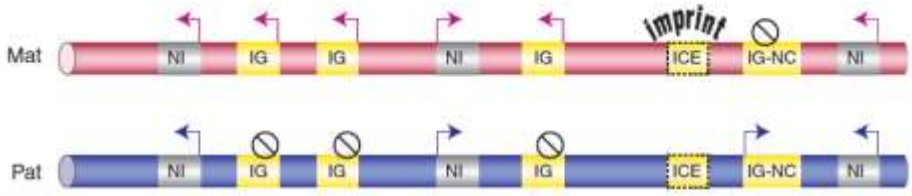


Figure 5. Imprinted genes are expressed from one parental allele and often clustered. Most imprinted genes (yellow) are found in clusters that include multiple protein-coding mRNAs (IG) and at least one noncoding RNA (IG-NC). Nonimprinted genes can also be present (NI in gray). The imprinting mechanism is *cis* acting and imprinted expression is controlled by an imprint control element (ICE) that carries an epigenetic imprint inherited from one parental gamete. One pair of diploid chromosomes is shown: the pink is of maternal origin and the blue of paternal origin. Arrow, expressed gene; slashed circle, repressed gene.

Second, the ICE deletion causes loss of imprinted expression only when deleted from the parental allele expressing the lncRNA. Table 1 shows that in three clusters (Igf2r, Kcnq1, and Gnas) the lncRNA promoter sits in an intron of one of the imprinted mRNAs, whereas in the remaining clusters the lncRNA promoter is separated, but lies close to the imprinted mRNA genes. This close intermingling of active and silent genes in an imprinted cluster indicates that the silencing and activating mechanisms affecting imprinted genes do not spread and may be restricted to the affected gene. In particular, the fact that the promoter of a silent lncRNA can reside in the intron of an actively transcribed gene indicates that silencing mechanisms may not even spread throughout the length of a gene, but may just be restricted to regulatory elements.

What is the role of the gametic DMR?

Despite the fact the gametic DMRs can be maternally or paternally methylated, experiments that deleted these elements have produced broadly similar results albeit with a few interesting exceptions (Fig. 6). For three clusters (Igf2r, Kcnq1, Dlk1), experimental deletion of the methylated gametic DMR produced no effect. In contrast, deletion of the unmethylated gametic DMR eliminated parental-specific expression causing a loss of lncRNA expression in cis and biallelic mRNA expression (Lin et al. 1995; Zwart et al. 2001; Fitzpatrick et al. 2002). Two clusters (Gnas and Pws) appear to contain more than one gametic DMR and show a more complex behaviour, yet they still share some similarities with the pattern presented in Figure 6 (Williamson et al. 2006). The Igf2 cluster, however, behaves differently: deletion of both the methylated and unmethylated gametic DMR causes changes in mRNA and lncRNA expression in cis (Thorvaldsen et al. 1998).

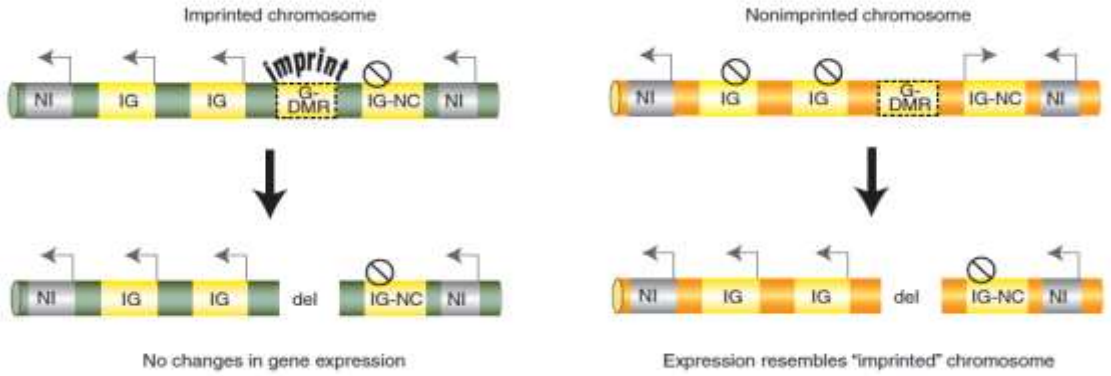


Figure 6. Imprinted expression is regulated by gametic DMRs (G-DMR). (*Left*) The effect of deleting the gametic DMR from the imprinted chromosome (green). (*Right*) The effect of deleting the G-DMR from the nonimprinted chromosome (yellow). In many imprinted clusters (e.g., *Igf2r*, *Kcnq1*, and *Dlk1*), experimental deletion of the G-DMR only affects the chromosome carrying the nonimprinted G-DMR. This results in a loss of repression of the imprinted protein-coding mRNA genes (IG) and a gain of repression of the imprinted lncRNA gene (IG-NC). Note that in some imprinted clusters (*Igf2* and *Pws*) that are not illustrated here, the methylated G-DMR appears also to be required for expression of some of the imprinted mRNAs in *cis*. del, deleted DNA; G-DMR, gametic differentially DNA-methylated region; NG, nonimprinted gene; arrow, expressed allele; slashed circle, repressed allele; imprint, epigenetic modification leading to a change in gene expression in *cis*.

The results from the above gametic DMR deletion experiments do not at first glance indicate a common function for gametic DMRs. However, an understanding of their exact function depends on knowing the position of the DMR with respect to the imprinted genes in each cluster. In the three clusters with the simplest pattern (Igf2r, Kcnq1, and Dlk1), the gametic DMR either contains or controls expression of the lncRNA, thus deletion of this element will clearly lead to loss of lncRNA expression. The gametic DMR in the Igf2 cluster, however, does not directly promote H19 transcription, but changes the interaction between Igf2 and H19 and their shared enhancers, and in this way regulates their expression. Despite these differences, in general, the unmethylated gametic DMR is implicated in all six clusters as a positive regulator of lncRNA expression, and the presence of the DNA methylation imprint is associated with repression of the lncRNA. The conclusion from the data obtained from deletion of gametic DMRs clearly identifies these regions as an ICE, whose activity is regulated by DNA methylation.

The Role of DNA Methylation in Genomic Imprinting:

The identification of the first three endogenous imprinted genes in 1991 enabled investigators to study how the cell's epigenetic machinery marked an imprinted gene with its parental identity. The first and most easily testable candidate was DNA methylation, a modification in mammals that covalently adds a methyl group to the cytosine residue in CpG dinucleotides. DNA methylation is acquired through the action of de novo methyltransferases and maintained in situ each time the cell divides by the action of maintenance methyltransferases (see Li and Zhang 2014).Hence, this modification fulfils the criteria outlined in Fig. 3 for a parental identity mark or "imprint" because (1) it can be established in either the sperm or oocyte by de novo methyltransferases that act only in one gamete, (2) it can be stably propagated at each embryonic cell division by a maintenance methyltransferase, and (3) it can be erased in the germline to reset the imprint in the next generation, either by passive demethylation (DNA replication followed by the failure to undergo maintenance methylation) or through the action of a demethylating activity (possibly through conversion of 5-methylcytosine to 5-

hydroxymethylcytosine by the ten-eleven translocation family of enzymes or through excision of 5-methylcytosine by the DNA repair machinery. DNA methylation could potentially perform two different functions in genomic imprinting. It could act as the imprinting mark by being acquired de novo only by the chromosomes in one gamete. It could also serve to silence one of the parental alleles because DNA methylation is associated with gene repression (Li and Zhang 2014). To determine which function it has, it is first necessary to show that DNA methylation is present only on one parental chromosome (i.e., that it is a DMR). Second, it is necessary to identify which imprinted gene in the cluster and which regulatory sequences are marked by DNA methylation.

The location of methylation marks on a promoter, or on distant positive or negative regulatory elements will have different consequences for gene expression. Finally, it is necessary to identify when the DMR forms during development. If it forms during gametogenesis and is continuously maintained in place in somatic cells (known as a gametic DMR), it may serve as the imprinting mark. If, however, it is placed on the gene after the embryo has become diploid when both parental chromosomes are in the same cell (known as a somatic DMR), it is unlikely to serve as the identity mark, but may serve to maintain parental specific silencing.

Parental allele-specific DNA methylation has been found at most imprinted clusters that have been examined. For example, the Igf2 cluster has a gametic DMR located 2 kb upstream of the H19 lncRNA promoter that is methylated only in the paternal gamete and is maintained thereafter in all somatic tissues (Bartolomei et al. 1993).A similar gametic DMR was identified covering the promoter of the Airn lncRNA, present only on the silent maternal gene copy, and acquired in the female gamete (Stoger et al. 1993). Surprisingly, gametic DMRs were not identified at the promoters of the principal imprinted protein-coding genes in these clusters (respectively, Igf2 and Igf2r). Instead, the silenced Igf2 promoter is free of DNA methylation, whereas the silenced Igf2r promoter lies within a somatic DMR that is placed after fertilization (Sasaki et al. 1992; Stoger et al. 1993). Similar findings of gametic DMRs methylated on the chromosome carrying the silent copy of the imprinted lncRNA (Fig. 6) have been made for other well-studied imprinted gene clusters, including Pws, Kcnq1, Gnas, Dlk1, and Grb10. Somatic DMRs are relatively rare but have been reported for some imprinted clusters, which suggests that this type of epigenetic modification plays a limited role in maintaining imprinted gene expression. Deletions of gametic DMRs in mice result in complete loss of imprinting for multiple genes, thereby proving that this class of DMRs also serves as a major ICE for the whole cluster (Fig. 6). In contrast, deletion of the somatic DMRs affects expression of the adjacent imprinted gene, but imprinted expression is maintained by other genes in the cluster (Constancia et al. 2000; Sleutels et al. 2003).

Genome-wide deficiency in DNA methylation caused by mutations in the Dnmt gene family underscores the essential role of DNA methylation in regulating imprinted gene expression. Mutations in the de novo DNA methyltransferase Dnmt3a, the DNA methyltransferase stimulatory factor Dmnt3L, or the Dnmt1 maintenance DNA methyltransferase generate DNA methylation deficient embryos that show alterations in imprinted gene expression. The type of perturbations shown for four imprinted clusters (Igf2, Igf2r, Kcnq1, and Dlk1) indicates that DNA methylation is generally acting to suppress the action of the gametic DMR. Thus, in the absence of DNA methylation, the gametic DMR cannot function appropriately (i.e., cannot

silence the lncRNA). As a consequence, the lncRNA is ab-errantly expressed and several imprinted protein-coding genes, including Igf2, Igf2r, Kcnq1, and Dlk1, become repressed on both parental chromosomes. This indicates that these mRNA genes require epigenetic modification of a cis-regulatory element to be expressed. Notably, the H19 lncRNA that is normally only expressed on the chromosome carrying the unmethylated gametic DMR becomes expressed on both parental chromosomes. Some exceptions to this general pattern have been reported for genes that show imprinted expression only in the placenta (Lewis et al. 2004).

Are other types of epigenetic modification used as gametic imprints? Given the sheer abundance of epigenetic mechanisms acting to modify genetic information in the mammalian genome, DNA methylation is unlikely to be the only imprinting mechanism. Histone modifications that affect chromatin activity states are also likely candidates for parental imprints because they could fulfil many of the prerequisites shown in Fig. 3. In one example, the Polycomb group protein known as EED (part of the PRC2 complex that catalyses methylation of H3K27, i.e., histone H3 at lysine 27) has been shown to affect a few paternally repressed genes in the placenta. The effects of Eed mutation on genomic imprinting, however, are relatively minor compared to that of DNA methylation (Mager et al. 2003). In another example, the EHMT2 histone methyltransferase acting specifically on H3K9 is required to repress a few imprinted genes, but also only in the placenta (Nagano et al. 2008). Thus, evidence to date suggests that histone modifications and modifying enzymes play a minor role in genomic imprinting.

Although much is known about the identity and epigenetic modifications of gametic DMRs, much less is known about how these sequences are chosen for methylation in the gametes. To date, many more maternally than paternally methylated gametic DMRs have been identified (Bartolomei and Ferguson-Smith 2011). The maternally methylated DMRs are methylated during oocyte growth and the paternally methylated DMRs are methylated prenatally in pro-spermatogonia (Fig. 7) (Lucifero et al. 2002). For maternal gametic DMRs, a sequence comparison of known gametic DMRs reveals no striking sequence conservation although some contain a series of direct repeats that may adopt a secondary structure that attracts DNA methylation (Neumann et al. 1995). The tandem direct repeats in the Igf2r cluster gametic DMR have, for instance, been shown to be essential for oocyte-specific DNA methylation (Koerner et al. 2012). Those in the Kcnq1 cluster gametic DMR, however, are not essential (Mancini-DiNardo et al. 2006). Another feature of maternal DMRs is that they are markedly CpG rich compared to the remainder of the genome. One idea for how these regions are recognized comes from the structural analysis of the complexed carboxy-terminal domains of DNMT3A and DNMT3L, which was obtained by X-ray crystallography (Jia et al. 2007).A tetrameric complex consisting of these two enzymes preferentially methylate a pair of CpGs that are 8–10 base pairs apart (Cheng 2014). Such spacing is found in maternally methylated, but not in paternally methylated imprinted loci. This CpG spacing, however, is widespread in the genome, questioning the specificity of such a mechanism or indicating that additional features are required (Ferguson-Smith and Greally 2007). Additional specificity has been suggested by the demonstration that DNMT3L interacts with the amino terminus of histone H3 if the H3K4 residue is unmethylated, and promotes local DNA methylation. Another factor contributing to the specificity of de novo DNA methylation at DMRs in the oocyte is

transcription across differentially methylated regions (Chotalia et al. 2009). Importantly, only protein-coding transcripts traversing the germline ICEs are thought to be involved in DNA methylation establishment. Although it is, as yet, unclear how this transcription may be attracting the DNA methylation machinery, it has been suggested that transcription across ICEs is required to establish or maintain open chromatin domains that are permissive for the establishment of DNA methylation. To investigate and define the mechanism further, it will be necessary to describe the temporal relationship between transcription and de novo DNA methylation in greater detail. Nevertheless, CpG spacing, posttranslational histone modifications, and transcription in oocytes could provide a starting point for the acquisition of maternal-specific DNA methylation imprints.

There is far less information regarding how paternal specific DNA methylation imprints are established in the male germline. Nevertheless, early experiments suggest there could be some similarities with the female germline. It has recently been shown that high transcriptional readthrough, predominantly from one strand, is detected at two paternal gametic DMRs in primordial germ cells, H19- DMD and IG-DMR, at the time of imprint establishment (Henckel et al. 2011). It also appears that maternal gametic DMRs, which are protected from DNA methylation, are enriched for H3 lysine 4 trimethylation (H3K4me3) in male primordial germ cells. One of the most mysterious questions in genomic imprinting is how the DNA methylation marks at imprinted genes escape the genome-wide reprogramming that occurs after fertilization, including the DNA demethylation that occurs in the preimplantation embryo and the subsequent wave of de novo DNA methylation (Fig. 7). It is likely that a combination of cis-acting sequences and trans-acting factors are mediating the protection. One maternal factor, PGC7/ STELLA, appears to have a general role in maintaining DNA methylation in the early mouse embryo through interactions with H3K9me2 (Nakamura et al. 2012). However, a factor that may be more specific for imprinted genes is ZFP57. Studies have shown that ZFP57mutations identified in transient neonatal diabetes patients are associated with defects in DNA methylation at multiple imprinted loci (Mackay et al. 2008). Additionally, Zfp57 null mice show embryonic lethality and loss of imprinting at many (but not all) loci (Li et al. 2008). More recently, it has been shown that ZFP57 binds to cofactor KAP1, which can then recruit other epigenetic regulators (Quenneville et al. 2011). Thus, sequence- and DNA methylation-dependent binding of ZFP57 could act as an anchor to specify allelic binding of KAP1, which would subsequently recruit other major repressive epigenetic regulators such as SETDB1, HP1, DNMT1, DNMT3A, and DNMT3B to the heterochromatic, silenced allele at imprinted loci. It is possible that other yet-to-be-identified proteins also maintain DNA methylation at imprinted loci in the early embryo.

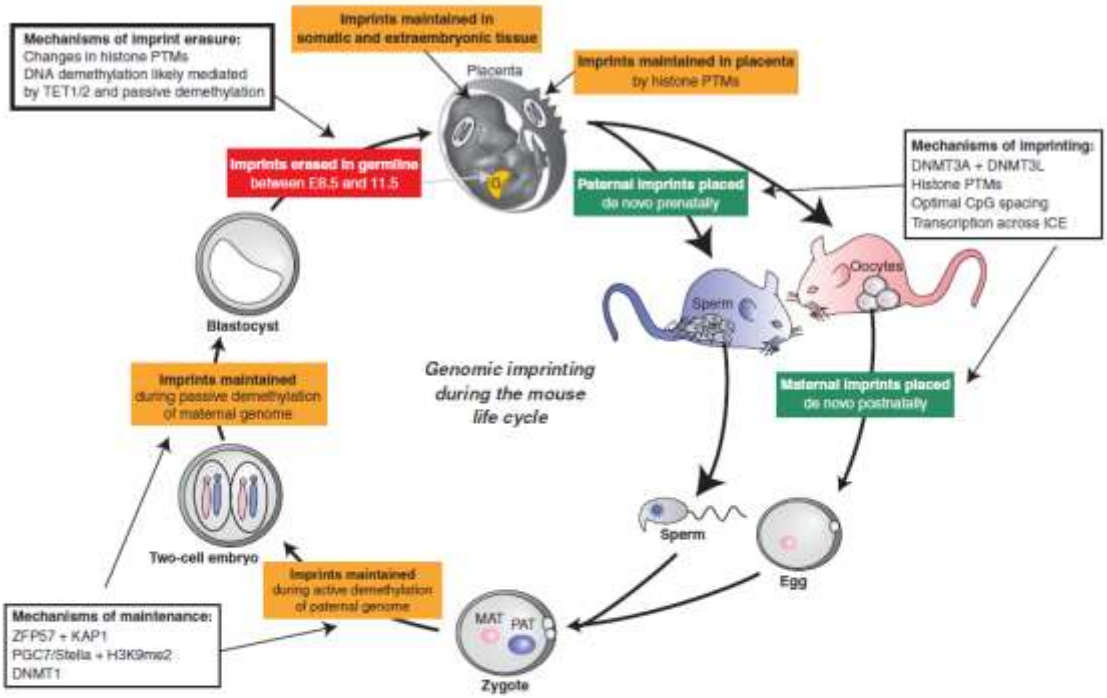


Figure 7. Establishment, maintenance, and erasure of genomic imprints in mouse development. In the germline, primordial germ cells (PGCs) undergo multiple changes in chromatin structure and DNA demethylation during migration into the genital ridge (gonad). Imprints are then acquired in a sex-specific manner in the germline (green shading). DNA methylation is targeted specifically to paternally and maternally DNA-methylated ICEs—prenatally in prospermatogonia and postnatally during oocyte maturation. These imprints are maintained despite global changes in DNA methylation after fertilization (orange shading): active demethylation of the paternal genome in the zygote and passive maternal demethylation in the preimplantation embryo. Candidates for protection of methylation regions include ZFP57 and PGC7/STELLA. De novo DNA methylation of the genome begins at the morula stage, during which time unmethylated alleles of imprinted genes must be protected. These imprints are maintained in somatic cells throughout the lifetime of the organism, whereas imprinting in extraembryonic tissues is thought to be less dependent on maintenance of DNA methylation. In the germline, imprints are erased and reset for the next generation (red shading). PTM, post-translational modification; MAT, maternal genome; PAT, paternal genome.

Two Types of cis-Acting Silencing Identified in Imprinted Gene Clusters:

Currently, two major classes of cis-acting silencing mechanisms are hypothesized to govern imprinting at various clusters: the insulator model applicable to the Igf2 cluster and the lncRNA-mediated silencing model applicable to the Igf2r and Kcnq1 clusters. Although not yet completely defined, most of the clusters in Table 1 incorporate aspects of one of the two models. The breakthrough that led to the definition of the insulator model at the Igf2 locus was the deletion of the gametic DMR (H19-DMD) that is located 2 kb upstream of the start of H19 transcription and 80 kb downstream of Igf2 (Fig. 8) (Thorvaldsen et al. 1998).

When deleted, H19 and Igf2 showed a loss of imprinting regardless of whether the deletion was inherited maternally or paternally, identifying this DMR as an ICE. It was subsequently shown that this ICE bound CTCF, a protein shown to mediate insulator activity at the beta-globin locus, and that the ICE itself functioned as an insulator (Bell and Felsenfeld 2000; Hark et al. 2000). In this context, an insulator s defined as an element that blocks enhancer and promoter interactions when placed between them. Thus, the model for imprinted gene expression at this locus is as follows: on the maternal allele, CTCF binds to the ICE and blocks the access of Igf2 and Ins2 to enhancers shared with the H19 lncRNA that are located downstream of the three genes. This thereby allows H19 exclusive access to the enhancers (Fig. 8). On the paternal allele, the ICE acquires DNA methylation in the male germline, preventing

CTCF from binding to it. Thus, on the paternal chromosome, Igf2 and Ins2 interact with the enhancers and are expressed from this chromosome. The presence of DNA methylation on the paternal ICE leads to secondary methylation of the H19 promoter by an unknown mechanism and it becomes silenced on the paternal chromosome. Although the insulator model is widely accepted, it is unclear how the insulator acts at this locus. One of the most widely held views is that CTCF interacts with DNA molecules in cis to insulate genes through the formation of chromatin loops. Moreover, it has been shown that cohesin interacts with CTCF to form these loops (Nativio et al. 2009). The involvement of CTCF in the insulator model has led to the identification of CTCF binding sites at other imprinted genes such as Rasgrf1, Grb10, and Kcnq1ot1, indicating that the insulator model may operate in other imprinted clusters. The lncRNA class of imprinting model may, however, be more common. The breakthrough that led to the identification of functional ncRNAs in imprinted clusters was an experiment that truncated the 108-kb Airn lncRNA to 3 kb (Sleutels et al. 2002). This shortened lncRNA retained imprinted expression and the Airn promoter retained imprinted DNA methylation—yet silencing of all three mRNA genes in the Igf2r cluster was lost (Fig. 8). lncRNA-mediated silencing has also now been shown to operate at the Kcnq1 cluster(Mancini-DiNardo et al. 2006), although in a tissue specific manner, suggesting that another mechanism such as one that uses insulators may also be involved at this cluster (Shin et al. 2008), and in the Gnas imprinted cluster (Williamson et al. 2011). At this time it is not precisely known how lncRNAs silence genes but many models are possible. Two possibilities arise from the sense–antisense overlap between an mRNA and the lncRNA that occurs in each cluster. The first possibility is that double-stranded RNA can form between them RNA and lncRNA and induce RNA-interference (RNAi) (described in Martienssen and Moazed 2014). Absence of the RNAi machinery, however, does not affect imprinted expression in the Kcnq1 cluster (Redrup et al. 2009). Thus, a second possibility is that this sense–antisense overlap causes a form of transcriptional interference of a promoter or an enhancer, which affects transcription from the mRNA promoter (Pauler et al. 2012). In this case, the first event could be silencing of the overlapped promoter or enhancer followed by accumulation of repressive chromatin that can spread and induce transcriptional gene silencing throughout the cluster. Evidence for this model comes from a series of recombinant endogenous chromosomes generated at the Igf2r/Airn locus in ES (embryonic stem) cells (Latos et al. 2012). The onset of allele-specific expression at this locus in the embryo can be recapitulated by ES cell differentiation, in which Igf2r is initially biallelically expressed, but the initiation of Airn expression results in Igf2r imprinting (Latos et al. 2009). To test whether Airn transcription or the lncRNA itself was required for Igf2r silencing, Airn was shortened to different lengths, with the result that silencing only required Airn transcription overlap of the Igf2r promoter, which interferes with RNA polymerase II recruitment (Latos et al. 2012). This model suggests that Airn acts predominantly through its transcription rather than as an lncRNA. It is, however, also possible that imprinted lncRNAs act by coating the local chromosomal region and directly recruit repressive chromatin proteins to the imprinted cluster, in a manner similar to that described for the action of the Xist lncRNA in X-chromosome inactivation (Brockdorff and Turner 2014). Evidence for a function of the lncRNA in recruitment of histone posttranslational modification machinery comes from experiments in placental tissues. RNA fluorescence in situ hybridization experiments showed that Airn and Kcnq1ot1 form RNA clouds at their site of transcription (Nagano et al. 2008;

Pandey et al. 2008; Terranova et al. 2008; Redrup et al. 2009). Terranova and colleagues show that these long ncRNAs are associated with a repressive histone compartment and Polycomb group proteins (Terranova et al. 2008). This nuclear compartment is also devoid of RNA polymerase II and exists in a three-dimensionally contracted state. Other studies on the Airn lncRNA go further in suggesting that the lncRNAs actively recruit repressive histone modifications (Nagano et al. 2008), but only in the placenta. In this latter case, Airn was shown to actively recruit the EHMT2 H3K9 methyltransferase. This resulted in the paternal-specific silencing of the Slc22a3 gene but not the Igf2r gene. These experiments indicate that lncRNA mediated silencing of imprinted genes may depend on different downstream mechanisms.

Importantly, other mechanisms of imprinted gene regulation are likely. For example, Wood and colleagues described a new imprinted locus (H13) in which alternative polyadenylation sites are used in an allele-specific manner (Wood et al. 2008). The H13 gene contains a maternally methylated internal CpG island that acquires DNA methylation in oocytes (it has not been tested for ICE activity yet). Hypermethylation of this CpG island ensures synthesis of the full length and functional H13 gene transcript from the maternal chromosome. Experiments showed that the unmethylated CpG island on the paternal allele allowed transcription from the promoter for the Mcts2 retrogene. Mcts2 expression, in turn, correlates with the premature polyadenylation of H13 and, hence, expression of truncated H13 transcripts. This locus raises the possibility that other less widely used mechanisms of genomic imprinting will be identified once the full catalogue of imprinted genes is elucidated.

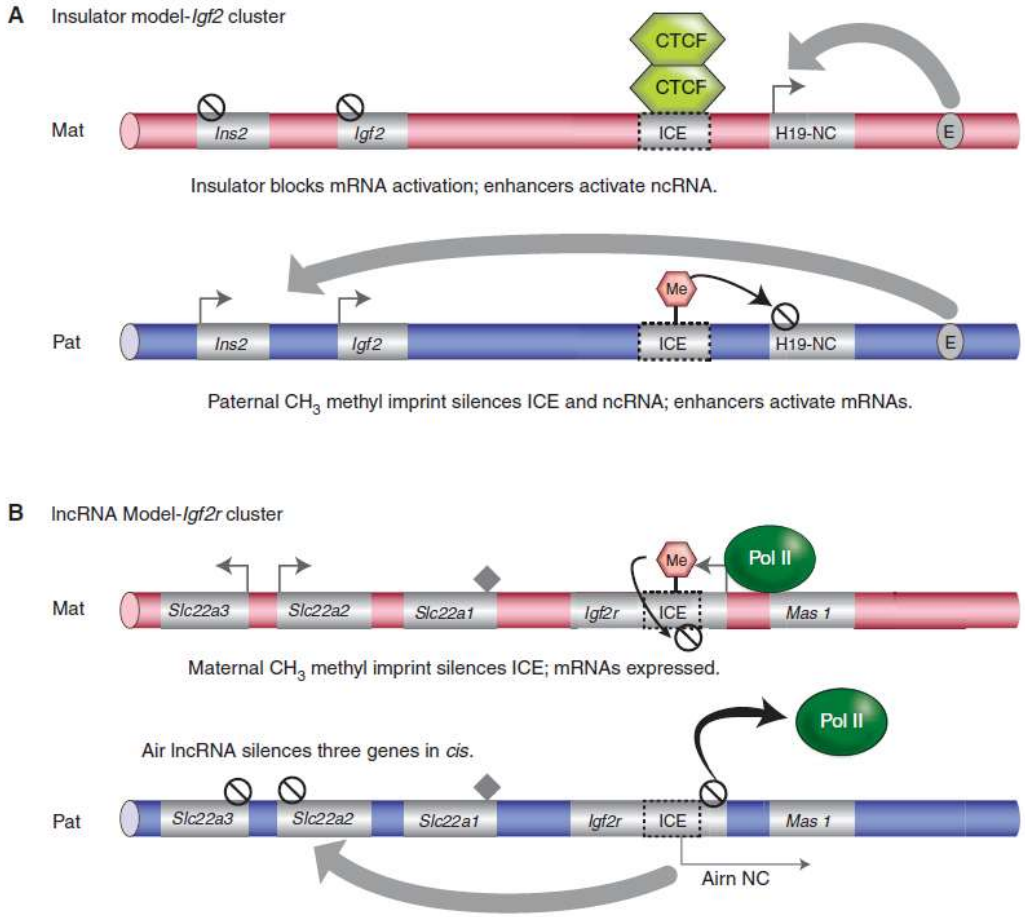


Figure 8. Two *cis*-acting silencing mechanisms at imprinted gene clusters. (*A*) Insulator model for the *Igf2* cluster. The expression pattern for endoderm is shown. On the maternal chromosome, the unmethylated ICE binds the CTCF protein and forms an insulator that prevents the common endoderm enhancers (E) from activating *Igf2* and *Ins2*. Instead the enhancers activate the nearby *H19* lncRNA promoter. On the paternal chromosome, the methylated ICE cannot bind CTCF and an insulator does not form; hence the *Igf2* and *Ins2* mRNA genes are expressed only on this chromosome. The *H19* lncRNA is methylated, most likely because of spreading from the 2-kb distant methylated ICE, and silenced. (*B*) lncRNA model for the *Igf2r* cluster. The expression pattern for placenta is shown. On the maternal chromosome, the methylated ICE contains the *Airn* lncRNA promoter that is directly silenced by the DNA methylation imprint. The *Igf2r*, *Slc22a2*, and *Slc22a3* mRNA genes are expressed only on this chromosome. *Mas1* and *Slc22a1* are not expressed in placenta (filled diamond). On the paternal chromosome, the Airn lncRNA promoter lying in the unmethylated ICE is expressed and silences *Igf2r* (in part by kicking off RNA polymerase II), *Slc22a2*, and *Slc22a3* in *cis*. Note that in both models, the DNA methylation imprint silences the lncRNA and permits mRNA expression. ICE, imprint control element; gray arrow, expressed allele of an imprinted gene; slashed circle, repressed allele of an imprinted gene; thick gray arrows, long distance effect in *cis*.

Genomic Imprinting - A model for mammalian epigenetic regulation:

Studying genomic imprinting has an advantage over other mammalian epigenetic gene regulation models because both the active and inactive parental allele reside in the same nucleus and are exposed to the same transcriptional environment (Bartolomei 2009; Barlow 2011). As a result, any epigenetic difference between the two parental alleles is more likely to correlate to their transcriptional state in contrast to “before and after” epigenetic systems, in which epigenetic changes may also reflect the altered differentiation state of the cell. The presence of both the active and silent parental allele in the same nucleus makes genomic imprinting an ideal system to study epigenetic gene regulation. At the same time, it imposes a difficulty because it is necessary to first distinguish between the parental alleles so that specific

features associated with gene activity and silencing can be attributed to the right parental allele. This difficulty has been largely overcome in the mouse by the development of model systems that allow the maternal and paternal chromosomes to be distinguished (Fig. 1). Despite the fact that epigenetic gene regulatory mechanisms are highly conserved in evolution, there are likely to be differences that relate to the type of genome organization for each organism. The mammalian genome shows an unusual organization that intersperses genes with high copy number repeats (also known as transposable elements). This greatly increases the length of most genes as well as the distance between adjacent genes. This contrasts with other model organisms such as yeast, nematodes, plants, and Drosophila, whose genomes show a tendency toward remaining repeat-free or, at least to separate repeats from genes. How can genomic imprinting contribute to an understanding of mammalian epigenetics? Although the characterization of imprinted gene clusters is far from complete, they clearly have the potential to provide information about how genes are controlled in local regions or domains. To date, imprinted gene clusters have already provided examples of cis-acting DNA sequences that are regulated by DNA methylation, genes that are silenced by default in the mammalian genome and require epigenetic activation to be expressed, long range regulatory elements that can act as insulators, and unusual lncRNAs that silence large domains of genes in cis. Time will tell whether these types of epigenetic regulatory mechanisms are unique to imprinted clusters or whether they can also be found regulating expression of nonimprinted genes in the mammalian genome.

Conclusion:

Genomic imprinting has been the focus of intense interest since the discovery of the first imprinted genes in mammals in 1991. Whereas early experiments relied on molecular and genetic strategies to identify imprinted genes, high throughput technology on polymorphic individuals is allowing the complete determination of imprinted genes (Deveale et al. 2012) and regions containing parental-specific DNA methylation (Xie et al. 2012). These experiments are indicating that most genes showing ubiquitous imprinted expression have already been identified.

However, it is possible that some genes showing tissue specific imprinted expression remain to be identified (Prickett and Oakey 2012). Some questions still await conclusive answers, particularly those concerning why mammals alone among vertebrates use imprinted genes to regulate embryonic and neonatal growth. This lack of knowledge contrasts with the extensive progress during the intervening 20 years on elucidating the epigenetic mechanisms controlling imprinted expression in mammals. From this information, we think we understand the general principles of how the imprinting mechanism operates at imprinted gene clusters, although all the details are still not clear. At this stage, it is clear that genomic imprinting uses the cell's normal epigenetic machinery to regulate parental-specific expression, and that everything is set in motion by restricting this machinery in the gamete to just one parental allele. Although there are general similarities in the mechanism controlling imprinted expression at different gene clusters, it is not yet understood how many variants of this mechanism exist in the mammalian genome. In the future, it will also be of interest to determine to what degree nonimprinted genes are controlled by the epigenetic mechanisms described for imprinted gene clusters. Ultimately, transferring this knowledge for therapeutic use in humans, for example,

by inducing re-expression of the silent parental alleles in patients with the Prader-Willi and Angelman syndromes to ameliorate their symptoms would be of great benefit. An understanding of the way the cell controls epigenetic information is of increasing importance, with the realization that epigenetic regulation can also be disturbed in cancers (Jones and Baylin 2014), in assisted reproductive technologies and also in the aging process (Rando and Chang 2012; Berger and Sassone-Corsi 2014). An improved understand ing of genomic imprinting will undoubtedly continue to provide an important model to discover how the mammalian genome uses epigenetic mechanisms to regulate gene expression.

Probable questions:

1. What is Genomic imprinting? How it affect gene expression?
2. How does a gametic imprint control imprinted expression?
3. What is the function of genomic imprinting in mammals?
4. What is the role of the gametic DMR?
5. Discuss the Role of DNA Methylation in Genomic Imprinting.
6. Briefly discuss two types of cis-Acting Silencing identified in imprinted Gene Clusters.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

Unit-VI

Nuclear transplantation and the reprogramming of the genome. epigenetics and human disease, epigenetic determinants of cancer

Objective: In this unit we will discuss on nuclear transplantation and the reprogramming of the genome. We will also discuss epigenetics and human disease and also epigenetic determinants of cancer.

Nuclear Transplantation:

Nuclear transplantation is a method in which the nucleus of a donor cell is relocated to a target cell that has had its nucleus removed (enucleated). Nuclear transplantation has allowed experimental embryologists to manipulate the development of an organism and to study the potential of the nucleus to direct development. Nuclear transplantation, as it was first called, was later referred to as somatic nuclear transfer or cloning.

Yves Delage first wrote about nuclear transplantation in 1895, speculating that if one were to replace an egg nucleus with another egg's nucleus, full development would occur. Later in 1938, Hans Spemann suggested an experiment whereby, using technologies not yet available to him, one could remove the nucleus of an egg and replace it with a different nucleus extracted from a developed cell. Thomas King and Robert Briggs were the first to perform experimental nuclear transplantation. The technique was soon after used by John Gurdon and eventually led to the first clone of a mammal, "Dolly" the sheep, by Ian Wilmut in 1996.

Nearly fifteen years after Spemann wrote about the possibility of nuclear transplantation, Briggs and King, using northern leopard frogs (*Rana pipiens*), performed the first nuclear transplantation experiment. They transplanted the nucleus from an early stage embryo to an unfertilized egg that had been enucleated. The egg cell was pricked with a clean glass needle in order to induce a fertilization-like response. The faux activation of fertilization allowed for extraction of the nuclear material inside while also activating the host egg cell. Meanwhile, the nucleus of a donor cell was extracted and then inserted into the newly enucleated and activated egg cell. That process induced development of the host egg according to the instructions of the newly inserted nucleus, resulting in the formation of an organism with the same genetic material as the donor cell, or a clone. Briggs and King continued to examine the potential of differentiated cells throughout the 1950s. They found that if the donor nucleus was extracted later in development, the potential of directing full development in the activated egg cell was greatly reduced. After the Briggs and King experiments it was generally accepted that the nuclear material in developing cells slowly loses its potential for full development.

That view was challenged in 1958 when Gurdon's experiments with African claws frogs (*Xenopus laevis*) produced fully developed frogs from the transferred nucleus of cells much later in development. Gurdon allowed the cloned frogs to develop to sexual maturity and was

then able to mate two sexually mature clones, suggesting that the donor nuclei were able to fully redirect development. Gurdon's experiments were widely accepted by the scientific community but questions remained for several decades. Scientists were concerned about whether the nucleus of the host egg cell was truly enucleated. The question of whether remnants of the host egg cell or the inserted nucleus directed development remained unanswered from 1958 to 2002, despite many attempts by Gurdon to prove it was the inserted nucleus. In 2002, however, Konrad Hochedlinger and Rudolf Jaenisch published an experiment using nuclear transplantation of mature white blood cells to generate mouse clones. Hochedlinger and Jaenisch were able to show that the inserted nucleus induced development in the host egg cell.

Although experimental embryologists continued to use nuclear transplantation to create clones of several species, Ian Wilmut's cloning experiment in 1996 was a controversial and widely publicized cloning experiment. Dolly was cloned using the nucleus of a mammary gland cell from an adult sheep and transplanting it into an enucleated egg cell from another sheep. The activated egg cell was then transferred into a third surrogate sheep that carried Dolly to term. Dolly died at the age of to six due to lung disease and severe arthritis, and although her death was not attributed to the fact that she was a clone, many believe that the relationship between telomeres and aging was the reason for her demise.

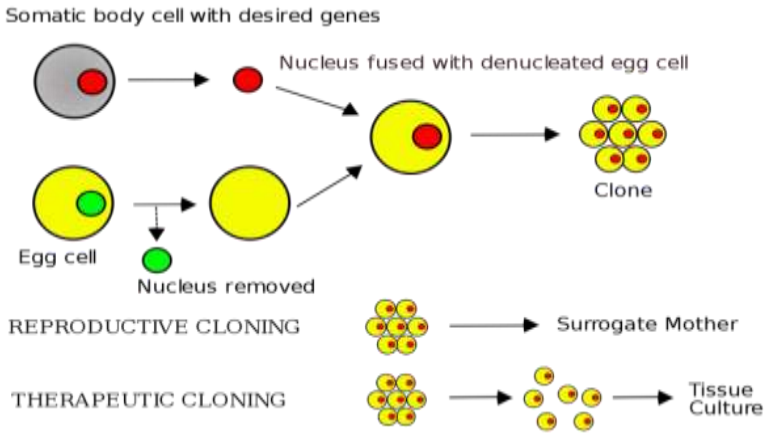
Nuclear transplantation may have begun as a subtle idea in the late 19th and early 20th centuries, but it evolved into a feasible and widely used process by experimental embryologists in the late 1990s. The cloning of Dolly the sheep worried many about the possibility of human cloning and the moral boundaries of modern advances in science. In the context of the embryonic stem cell discourse of the late 1990s and early twenty-first century, somatic nuclear transfer has been contrived into moral arguments about rights of the human embryo. Furthermore, nuclear transplantation has spurred ethical discussion on the value of a human life during all stages of development. Many scientists have abandoned the methods involved in nuclear transplantation and have adopted methods set forth by Shinya Yamanaka in his experiments involving induced pluripotent stem cells.

Brief Conventional Process:

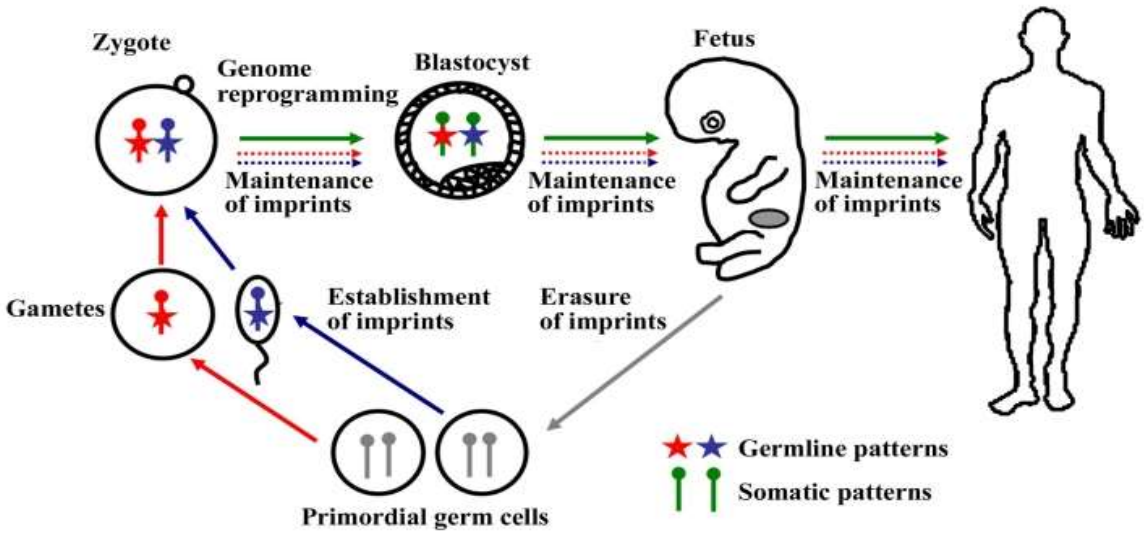
Nuclear Transfer Cloning:

Also known as "somatic cell nuclear transfer" (SCNU), the process of nuclear transfer cloning requires two cells. The somatic cell, the cell collected from the animal that is to be cloned (the genetic donor), is any cell other than an egg or sperm cell but contains the complete DNA (Deoxyribonucleic Acid) or genetic blueprint. This can be easily obtained from a skin biopsy performed by a veterinarian. The second cell required is the egg cell, which is collected from any female of the same species (the egg donor). In the figure below, the somatic cell is in grey and the egg cell, in yellow. Once both cells are obtained, the cloning process can begin. As shown in the diagram, the nucleus (green circle) of the egg cell is extracted and discarded of. By the doing this, it removes the egg cell of the genetic information of the original host, as the genes are contained within the nucleus. As the genetic information from the somatic cell is needed, the same is done; the nucleus of the somatic cell (red circle) is extracted, and the

resulting egg cell and nucleus are then "fused" together through electricity, resulting in the egg cell containing the DNA of the genetic donor. The cell is then stimulated, which then causes it to divide, just as any zygote (fertilised egg) would during reproduction, as shown in the part of figure 1 labelled "Clone". The egg is then placed in a culture medium, a liquid or gel designed to support growth micro-organisms or cells. The cellular division continues over several days until the blastocyst (early stage embryo) is formed. Within a week, an embryo transfer specialist inserts the blastocyst into a surrogate mother. Once it has fully developed, the mother gives birth to a clone of the genetic donor.



The concept of nuclear transfer cloning was created in 1928 by a German embryologist by the name of Hans Spemann. In his initial experiments, Spemann had transferred salamander embryonic cell nucleic into egg cells. It was only decades later that the embryologist conceived the concept of generating clones by transferring nuclei from differentiated cells into enucleated egg cells, nuclear transfer cloning. At the time, Spemann thought this process impossible, due to the fact that the microsurgical tools necessary to perform such delicate procedure without causing damage to the genetic material or egg cells did not exist. Eventually in the 1990's, a team of scientists from Roslin Institute Scotland used the proposed process to produce sheep clones, and thus in 1996, Dolly was born (figure below). This process has helped make major strides in the area of stem cell research and nuclear reprogramming.



Basic principles of epigenetics:

DNA methylation and histone modifications The human genome contains 23,000 genes that must be expressed in specific cells at precise times. Cells manage gene expression by wrapping DNA around clusters (octamers) of globular histone proteins to form nucleosomes (Fig. 1A). These nucleosomes of DNA and histones are organized into chromatin. Changes to the structure of chromatin influence gene expression: genes are inactivated (switched off) when the chromatin is condensed (silent), and they are expressed (switched on) when chromatin is open (active) (Fig. 1B). These dynamic chromatin states are controlled by reversible epigenetic patterns of DNA methylation and histone modifications. Enzymes involved in this process include DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone acetylases, histone methyltransferases and the methyl-binding domain protein MECP2. Alterations in these normal epigenetic patterns can deregulate patterns of gene expression, which results in profound and diverse clinical outcomes.

The loss of normal DNA methylation patterns is the best understood epigenetic cause of disease, based on the initial studies during the 1980s that focused on X chromosome inactivation, genomic imprinting and cancer. DNA methylation involves the addition of a methyl group to cytosines within CpG (cytosine/guanine) pairs (Fig. 1A). Typically, unmethylated clusters of CpG pairs are located in tissue specific genes and in essential "housekeeping" genes, which are involved in routine maintenance roles and are expressed in most tissues. These clusters, or CpG "islands," are targets for proteins that bind to unmethylated CpGs and initiate gene transcription. In contrast, methylated CpGs are generally associated with silent DNA, can block methylation sensitive proteins and can be easily mutated. DNA methylation patterns are established and maintained by DNMTs, enzymes that are essential for proper gene expression patterns. In animal experiments, the removal of genes that encode DNMTs is lethal; in humans, overexpression of these enzymes has been linked to a variety of cancers. In addition to DNA methylation, changes to histone proteins orchestrate DNA organization and gene expression. Histone-modifying enzymes are recruited to ensure that a receptive DNA region is either accessible for transcription or that DNA is targeted for silencing (Fig. 1B). Active regions of chromatin have unmethylated DNA and have high levels of acetylated histones, whereas inactive regions of chromatin contain methylated DNA and deacetylated histones. Thus, an epigenetic "tag" is placed on targeted DNA, marking it with a special status that specifically activates or silences genes. These reversible modifications ensure that specific genes can be expressed or silenced depending on specific developmental or biochemical cues, such as changes in hormone levels, dietary components or drug exposures.

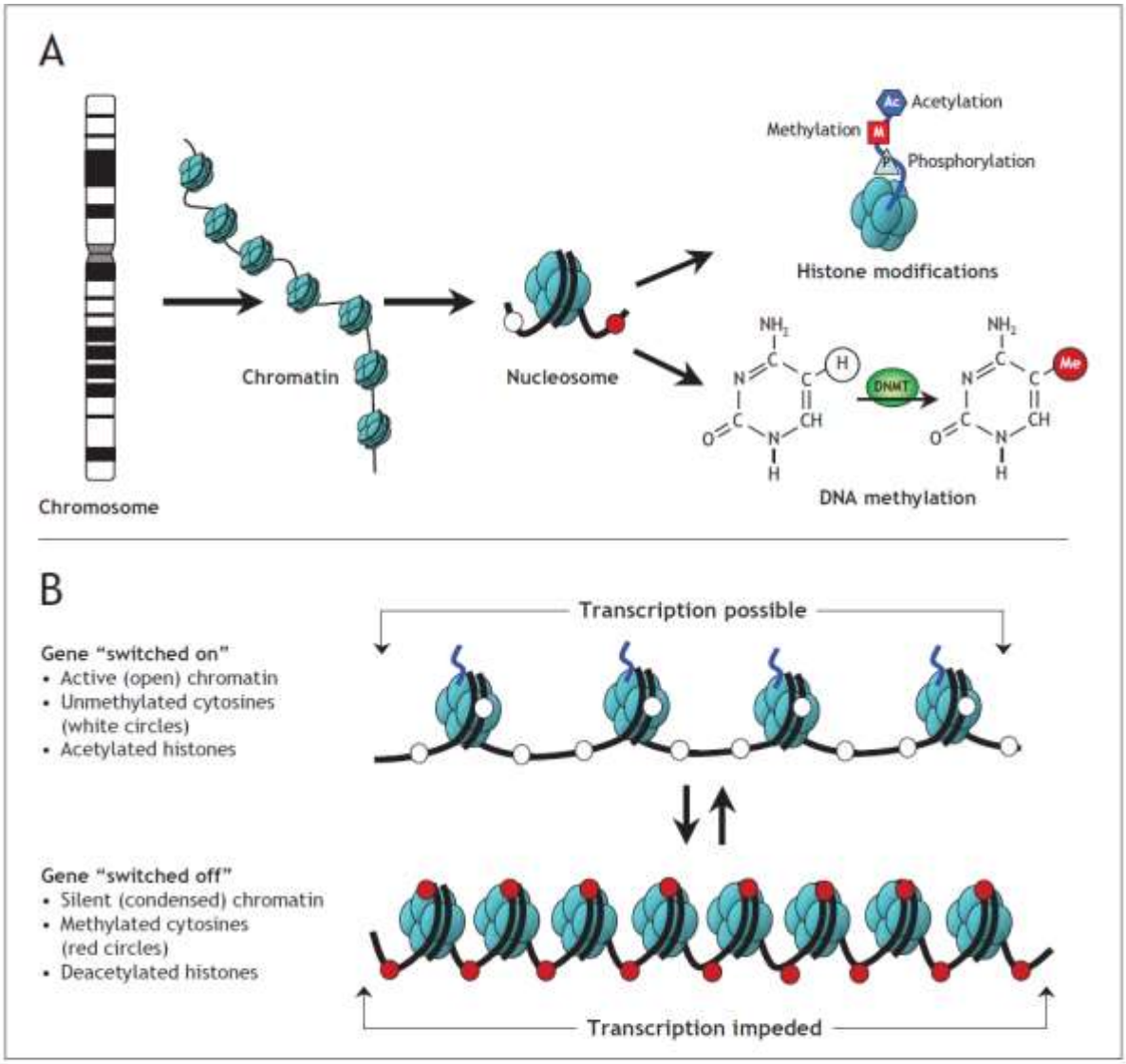


Fig 1: (A) Schematic of epigenetic modifications. Strands of DNA are wrapped around histone octamers, forming nucleosomes. These nucleosomes are organized into chromatin, the building block of a chromosome. Reversible and site-specific histone modifications occur at multiple sites through acetylation, methylation and phosphorylation. DNA methylation occurs at 5-position of cytosine residues in a reaction catalyzed by DNA methyltransferases (DNMTs). Together, these modifications provide a unique epigenetic signature that regulates chromatin organization and gene expression. (B) Schematic of the reversible changes in chromatin organization that influence gene expression: genes are expressed (switched on) when the chromatin is open (active), and they are inactivated (switched off) when the chromatin is condensed (silent).[3] White circles = unmethylated cytosines; red circles = methylated cytosines.

Genomic imprinting and imprinting disorders:

Genomic imprinting allows genes to "remember" whether they were inherited from the mother or the father so that only the maternally or paternally inherited allele is expressed. Imprinting is regulated by DNA methylation and histone modifications and is important in the context of a variety of developmental and paediatric disorders. Prader–Willi, Angelman and Beckwith–Weidemann syndromes best characterize congenital imprinting disorders. Prader–Willi and Angelman syndromes are caused by genetic and epigenetic errors to the same part of chromosome; errors inherited from the father result in Prader–Willi syndrome, and those inherited from the mother, Angelman syndrome. Beckwith–Wiedemann syndrome is caused by genetic or epigenetic mutations resulting in loss of imprinting on chromosome 11. Besides

gene-specific imprinting effects, global imprinting changes can occur in embryos that completely lack one parental genome. For example, spontaneous activation of oocytes in situ leads to ovarian teratomas that lack a paternal genome. In contrast, complete hydatidiform moles have been found that lack a maternal genome and an embryo and exhibit hyperproliferation of trophectoderm tissues, with the potential of forming choriocarcinoma. The recessive disorder "familial biparental complete hydatidiform mole" also leads to recurrent development of moles when maternal specific imprints fail to be established during oogenesis. Interestingly, imprinting effects that appear to target trophoblast cells have been recently implicated as a cause of preeclampsia.

Aging and epigenetics:

Both increases and decreases in DNA methylation are associated with the aging process, and evidence is accumulating that age-dependent methylation changes are involved in the development of neurologic disorders, autoimmunity and cancer in elderly people.88 Methylation changes that occur in an age-related manner may include the inactivation of cancer related genes. In some tissues, levels of methylated cytosines decrease in aging cells, and this demethylation may promote chromosomal instability and rearrangements, which increases the risk of neoplasia. In other tissues, such as the intestinal crypts, increased global hypermethylation may be the predisposing event that accounts for the increased risk of colon cancer with advancing age.

Table 1: Associations between epigenetic modifications and human diseases and conditions

Disease/condition	Gene	Biological process	Disease/condition	Gene	Biological process
Cancer			Neurologic		
Bladder	Multiple genes	Hypermethylation[20]	Schizophrenia	*RELN*	Hypermethylation[46,47]
Brain (glioma)	*RASSF1A*	Hypermethylation[28,29]	Bipolar disorder	*11p?*	Unknown[48]
Brain (glioblast)	*MGMT*	Hypermethylation[30]	Memory formation	Multiple genes	Hypo-, hypermethylation[49]
Breast	*BRCA1*	Hypermethylation[31]	Lupus	Retroviral DNA	Hypomethylation[50]
Breast	Multiple genes	Hypermethylation[32,33]	Cardiovascular		
Cervix	*p16*	Hypermethylation[34]	Atherosclerosis	Multiple genes	Hypo-, hypermethylation[19,51]
Colon	Multiple genes	Hypermethylation[20]	Homocysteinemia	Multiple genes	Hypomethylation[52]
Colorectal	L1 repeats	Hypomethylation[35]	Vascular endothelium	*eNOS*	Hypomethylation[53]
Esophagus	*CDH1*	Hypermethylation[20]	Imprinting and pediatric syndromes		
Head/neck	*p16, MGMT*	Hypermethylation[20]	PWS or AS	15q11-q13	Imprinting[54]
Kidney	*TIMP-3*	Hypermethylation[20]	BWS	11p15	Imprinting[55]
Leukemia	p15	Hypermethylation[20]	SRS	Chromosome 7	Imprinting[56]
Liver	Multiple genes	Hypermethylation[36]	UPD14	14q23-q32	Imprinting[57]
Lung	*p16, p73*	Hypermethylation[20]	PHP, AHO, MAS	20q13.2	Imprinting[58]
Lymphoma	*DAPK*	Hypermethylation[20]	Rett syndrome	*MECP2*	Mutation[59]
Myeloma	*DAPK*	Hypermethylation[37]	ICF syndrome	*DNMT3B*	Mutation[60]
Ovary	*BRCA1*	Hypermethylation[38]	ATRX	*ATRX*	Chromatin structure[61]
Ovary	*Sat2*	Hypomethylation[39]	FraX	Triplet repeat	Silencing[62]
Pancreas	*APC*	Hypermethylation[20]	FSHD	3.3 kb repeat	Chromatin structure[63]
Pancreas	Multiple genes	Hypomethylation[40]	Reproductive		
Prostate	*BRCA2*	Hypermethylation[20,41]	Ovarian teratoma	No paternal genome	Imprinting[64]
Rhabdomyosarcoma	*PAX3*	Hypermethylation[42]	CHM	No maternal genome	Imprinting[65]
Stomach	*Cyclin D2*	Hypomethylation[43]	BiCHM	Maternal genome	Imprinting[65]
Thymus	*POMC*	Hypomethylation[44]	Aging	Chromatin	Hypo-, hypermethylation[66]
Urothelial	Satellite DNA	Hypomethylation[45]			
Uterus	*hMLH1*	Hypermethylation[20]			

Note: PWS = Prader-Willi syndrome; AS = Angelman syndrome; BWS = Beckwith-Weidemann syndrome; SRS = Silver-Russell syndrome; UPD14 = uniparental disomy 14; PHP = pseudohypoparathyroidism; AHO = Albright hereditary osteodystrophy; MAS = McCune-Albright syndrome; ICF = immunodeficiency, centromeric instability and facial anomalies; ATRX = α-thalassemia/mental retardation syndrome, X-linked; FraX = Fragile X syndrome; FSHD = facioscapulohumeral muscular dystrophy, CHM = complete hydatidiform mole, BiCHM = familial biparental CHM.

Immunity and related disorders:

The activation of the immune response involves stepwise epigenetic changes, which allow individual cells to mount a specific immune response that can be maintained over multiple cell generations.90,91 For example, shifts in both acetylation and methylation are required to coordinate DNA accessibility and permit recombination, thereby allowing cells to mount an immune response against a specific antigen. Recent reports suggest that loss of epigenetic control over this complex process contributes to autoimmune disease. Abnormal DNA methylation has been observed in patients with lupus whose T cells exhibit decreased extracellular signal-regulated kinase pathway signalling, decreased methyltransferase activity and hypomethylated DNA. Disregulation of this pathway apparently leads to overexpression of methylation-sensitive genes such as the leukocyte function-associated factor (LFA1), which causes lupus-like autoimmunity. Interestingly, LFA1 expression is also required for the development of arthritis, which raises the possibility that altered DNA methylation patterns may contribute to other diseases displaying idiopathic autoimmunity.

Neuropsychiatric disorders:

Recent reports have begun to address the role of epigenetic errors in the causation of complex adult psychiatric, autistic and neurodegenerative disorders (Table 1). Several reports have associated schizophrenia and mood disorders with DNA rearrangements that include the DNMT genes. DNMT1 is selectively overexpressed in gamma-aminobutyric acid (GABA)-ergic interneurons of schizophrenic brains, whereas hypermethylation has been shown to repress expression of Reelin (a protein required for normal neurotransmission, memory formation and synaptic plasticity) in brain tissue from patients with schizophrenia and patients with bipolar illness and psychosis. In addition, the HDAC inhibitor valproic acid has been shown to prevent Reelin promoter hypermethylation in a mouse model of schizophrenia. A role for aberrant methylation mediated by folate levels has been suggested as a factor in Alzheimer's disease; however, there is contradictory evidence regarding hypomethylation and overexpression of the presenilin-1 gene that is involved in synaptic plasticity, long-term memory and neuronal survival. As well, some preliminary evidence supports a model that incorporates both genetic and epigenetic contributions in the causation of autism. Autism has been linked to the region on chromosome 15 that is responsible for Prader–Willi syndrome and Angelman syndrome. Findings at autopsy of brain tissue from patients with autism have revealed deficiency in MECP2 expression that appears to account for reduced expression of several relevant genes. These results suggest that MECP2 deficiency plays a role in chromosome organization in the developing brain in autism, Rett syndrome and several other neurodevelopmental disorders. There may be a role for epigenetics in the diagnosis and treatment of complex neuropsychiatric disorders in the future.

Table 4. Summary of epigenetic aberrations reported in mental diseases

Disease	Epigenetic change (tissues)
Fragile X syndrome	Hyper-methylation at the FMR-1 gene with an expanded (CCG)n repeat
Huntington	Histone modification in HDACs and histone KDM5D/Kdm5d
Rett syndrome	Mutation in the gene encoding *MeCP2*
Autistic patients and their parents	Abnormal trans-methylation, trans-sulfuration metabolism, genome-wide DNA hypo-methylation and elevated blood homocysteine level (blood)
Down syndrome	miR-99a, let-7c, miR-125b-2, miR-155, and miR-802 up-regulation
SCZ	DNA hyper-methylation of the RELN promoter and SOX10 promoter (brain)
SCZ and BD	DNA hypo-methylation of the MB-COMT promoter (brain)
SCZ	Histone 3 lysine 4 hypo-methylation at the GAD1 promoter due to mixed-lineage leukemia 1 gene dysfunction (brain)
SCZ (male)	DNA hyper –methylation of the WDR18 gene (brain)
SCZ (male)	Global DNA hypo-methylation (blood)
SCZ & Psychotic BD	DNM T1 hyperexpression and increase in SAM content (corticalinter-neurons)
Bipolar ll	DNA hypo-methylation of *PPIEL* gene (blood)
BD (female)	Hypo-methylation of *RPL39* (brain)
Dementia	Hyper-methylation of circadian genes, *PER1* and *CRY1* (blood)
Alcoholism	DNA hyper-methylation of alpha synuclein promoter, HERP gene promoter and dopamine transporter gene (blood)

SCZ, schizophrenia; BD, bipolar disorders

Paediatric syndromes and epigenetics:

In addition to epigenetic alterations, specific mutations affecting components of the epigenetic pathway have been identified that are responsible for several syndromes: DNMT3B in the ICF (immunodeficiency, centromeric instability and facial anomalies) syndrome, MECP2 in Rett syndrome, 105 ATRX in ATR-X syndrome (a-thalassemia/mental retardation syndrome, X linked) and DNA repeats in facioscapulohumeral muscular dystrophy (Table 1). In Rett syndrome, for example, MECP2 encodes a protein that binds to methylated DNA; mutations in this protein cause abnormal gene expression patterns within the first year of life. Girls with Rett syndrome display reduced brain growth, loss of developmental milestones and profound mental disabilities. Similarly, the ATR-X syndrome also includes severe developmental deficiencies due to loss of ATRX, a protein involved in maintaining the condensed, inactive state of DNA. Together, this constellation of clinical paediatric syndromes is associated with alterations in genes and chromosomal regions necessary for proper neurologic and physical development.

Cancer and epigenetic therapies:

Cancer is a multistep process in which genetic and epigenetic errors accumulate and transform a normal cell into an invasive or metastatic tumour cell. Altered DNA methylation patterns

change the expression of cancer-associated genes (Table 1). DNA hypomethylation activates oncogenes and initiates chromosome instability, whereas DNA hypermethylation initiates silencing of tumour suppressor genes. The incidence of hypermethylation, particularly in sporadic cancers, varies with respect to the gene involved and the tumour type in which the event occurs. For example, p16ink4A promoter hypermethylation occurs in varying degrees (9%–49%) in as many as 15 cancer types; in contrast, BRCA1 hypermethylation is primarily associated with 10%–20% of sporadic breast and ovarian cancers.20 These epigenetic changes can be used in the molecular diagnosis of a variety of cancers. To date, epigenetic therapies are few in number, but several are currently being studied in clinical trials or have been approved for specific cancer types. Nucleoside analogues such as azacitidine are incorporated into replicating DNA, inhibit methylation and reactivate previously silenced genes. Azacitidine has been effective in phase I clinical trials in treating myelodysplastic syndrome and leukaemia characterized by gene hypermethylation. For example, 54% of patients with chronic myelogenous leukaemia resistant to imatinib exhibited a complete or partial hematologic response, and 46% had a major or minor cytogenetic response to 5-aza-2'-deoxycytidine. The antisense oligonucleotide MG98 that downregulates DNMT1 is showing promising results in phase I clinical trials and in targeting solid tumours and renal cell cancer. Molecular analysis of biopsies of head and neck cancer following MG98 treatment revealed demethylation of 2 methylated tumour suppressor genes and methylation of an oncogene. Similarly, small molecules such as valproic acid that downregulate HDACs are being used to induce growth arrest and tumour cell death. Combination epigenetic therapies (demethylating agents plus HDAC inhibitors) or epigenetic therapy followed by conventional chemotherapy (or immunotherapy) may be more effective since they reactivate silenced genes, including tumour suppressor genes, resensitize drug-resistant cells to standard therapies and act synergistically to kill cancer cells. The key challenge for the future will be to limit toxic effects in normal cells and ensure that these novel drug effects reach critical target genes in tumour cells.

Epigenetic modifications have a considerable effect on cancer. Hypermethylation of promoter regions in tumour suppressor genes can inactivate many tumour suppressor functions. Methylation levels also play an important role in cell divisions, DNA repair, differentiation, apoptosis, angiogenesis, metastasis, growth factor response, detoxification, and drug resistance. Such features have promoted huge advances in the early detection of cancer using methylation levels. For example, hypermethylation of promoter regions in APC and RASSF1A genes are considered as common epigenetic markers for early detection of cancer. Also, hypermethylation of TP53 promoter region has been reported as a common marker for evaluation of cancer development. There are also some other types of epigenetic changes in cancer. In recent years, dysregulation of miRNAs has been confirmed in breast cancer, which has a potential to be used as diagnostic biomarkers. Also, hyper- and hypo-methylation of several genes in breast cancer have been confirmed.
Microsatellite instability, chromosomal instability, and CpG island methylator phenotype have been identified as three major mechanisms affecting gene function in colorectal cancer (CRC). Microsatellite instability occurs in 15% of CRCs, which can result in instability phenotype by mutated or methylated mismatch repair genes[. In a comprehensive analysis of CRC tumours in Iranian patients, Brim et al. demonstrated a high microsatellite instability rate (18%). From

15 known methylation target genes, APC2, PTPRD, EVL, GPNMB, MMP2, and SYNE1 were found to be methylated in most samples, which can be potentially used as specific clinical and pathological markers of CRC in this population.

The pathogenesis of CRC has been reported to be controlled by miRNAs, which can act as regulators of oncogenic and tumour suppressor pathways, responsible for the development of cancer. It has been confirmed that different miRNAs can be useful as biomarkers and are potentially applicable in prognosis evaluation and the detection of CRC stages. It has been also observed that in the absence of O6-methylguanine-DNMTs activity as a DNA repair protein, the specific genes, such as K-ras and p53, might be accumulated by G-to-A transition. Furthermore, hypermethylation near the methylguanine-DNMT start codon in the specific locus is critical for cancer progression, which may have a prognostic value in CRC patients.

It has been indicated that miRNAs play an important role in many types of cancer: acute myeloid leukaemia, acute lymphocytic leukaemia, chronic myeloid leukaemia, chronic lymphocytic leukaemia, endometrial carcinoma, gastrointestinal cancer, lung cancer, bladder cancer, thyroid tumours, and oesophageal adenocarcinomas. Hence, the potential applications of miRNAs in diagnosis and prognosis of these cancers would be highlighted in the near future.

Isocitrate dehydrogenase 1 (IDH1) and IDH2 genes are frequently mutated in low-grade gliomas, de-novo acute myeloid leukaemia in adult and in the subsets of chondrosarcomas and lymphomas. Interestingly, high correlation between histone and DNA methylation phenotype in IDH mutant gliomas has been reported.

Table 1. Promoter methylation in different types of cancer

Cancer type	Gene	Promoter methylation	Reference
Breast	RARB2, MSH2, ESR1B, AKR1B1, COL6A2, GPX7, HIST1H3C, HOXB4, RASGRF2,TM6SF1, ARHGEF7, TMEFF2, RASSF1, BRCA1, STRATIFIN, RASSF1A	Hypermethylation	[102]
Gastric	RUNX3	Hypermethylation	[102]
Liver	CDKN2A	Hypermethylation	[102]
Esophageal	APC	Hypermethylation	
Colorectal	SEPT9, hMLH1, CDKN2A/p16, HTLF, ALX4, TMEFF2/HPP1, NGFR, SFRP2, NEUROG1, RUNX3,UBE2Q1	Hypermethylation	[103,104]
Lung	RARB2, RASSF1A, CHFR, STRATI-FIN, SHOX2, RASSF1A APC1	Hypermethylation	[102]

Table 2. Histone modifications in different types of cancer

Cancer type	Type of histone modification
Lung adenocarcinoma	Up-regulation of α-2 glycoprotein 1 in consequence of global histone acetylation[105]
Non-small cell lung	Global H3 deacetylation[106]
Gastric	Global H3K9 trimethylation[107] Silencing of RUNX3 in the consequence of increased H3K9 dimethylation and decreased H3 acetylation[108]
Prostate	Global H3K9, H3K18, and H4K12 acetylation and H4K3 and H3K4 dimethylation[109] Activation of PTEN, CYLD, p53, and FOX03a by modulating histone H3K9 methylation and deacetylation[110]
Colorectal	Global H3K9 deacetylation[111]
Pancreatic	Acetylation of histone H3 promoter region of C/EPBα[112]

Table 3. miRNA changes in different types of cancer[3,65,113]

Cancer type	Types of miRNA[(+)=up-regulation/(-)=down-regulation]
Oesophageal squamous cell carcinomas	miR-21(+)
Lung	miR-17-92 (+) miR-34c, miR-145, and miR-142-5p, let-7(-)
Primary head and neck squamous cell carcinoma	miR-1, miR-133a, miR-205, and led-7d(-) bsa-miR-21(+)
Gastric	miR-106a(+) miR-433 and miR-9(-)
Prostate	miR-135b and miR-194(+) miR-23b, miR-100, miR-145, miR-221, miR-222(-)
Melanoma	miR-182(+)
Hepatocellular	miR-18a(-)
Colorectal	miR-let 7g, miR-21, miR-20a, miR-17-19 family, miR31, miR 135, miR-181b, and miR 200c (+) miR-34, miR-let7, miR-143, miR-145, miR-133b, and miR-126(-)
Bladder	miR-2 23, miR-26b, miR-221, miR-103-1, miR-185, miR-23 b, miR- 203, miR 17-5p, miR-23, miR-205(+) miR-29c, miR-26a, miR-30c, miR-30e-5p, miR-45, miR-30a-3p, miR-133a, miR-133b, miR-195, miR-125b,and miR-199a (-)
Breast	miR-21, miR-155, miR-23, and miR-191(+) miR-205, miR-145, miR-10b, and miR-125b (-)

Epigenetic Determinants of Cancer

Epigenetic changes are present in all human cancers and are now known to cooperate with genetic alterations to drive the cancer phenotype. These changes involve DNA methylation, histone modifiers and readers, chromatin remodelers, microRNAs, and other components of chromatin. Cancer genetics and epigenetics are inextricably linked in generating the malignant

phenotype; epigenetic changes can cause mutations in genes, and, conversely, mutations are frequently observed in genes that modify the epigenome. Epigenetic therapies, in which the goal is to reverse these changes, are now one standard of care for a preleukemic disorder and form of lymphoma. The application of epigenetic therapies in the treatment of solid tumors is also emerging as a viable therapeutic route.

Cancer is caused by the somatically heritable deregulation of genes that control the processes governing when cells divide, die, and move from one part of the body to another. During carcinogenesis, genes can become activated in such a way that enhances division or prevents cell death (oncogene). Alternatively, genes can become inactivated so that they are no longer available to apply the brakes to these processes (tumour suppressor gene). It is the interplay between these two classes of genes that results in the formation of cancer. Tumour-suppressor genes (TSGs) can become inactivated by at least three pathways: (1) through mutations, in which their functions become disabled; (2) a gene can be completely lost and thus not be available to work appropriately (loss of heterozygosity); and (3) a gene can be switched off in a somatically heritable fashion by epigenetic changes, rather than by mutation of the DNA sequence. Epigenetic silencing can occur by deregulation of the epigenetic machinery at several different levels; it may involve inappropriate methylation of cytosine (C) residues in CpG sequence motifs that reside within control regions governing gene expression. Also, changes to histone posttranslational modifications (PTMs) or aberrations in the way histone-modifying enzymes function may occur. A change in a protein's ability to read histone marks, and hence bind to chromatin, or alterations in the way nucleosome-remodelling or histone exchange complexes function can result. Finally, changes in regulatory microRNA (miRNA) expression patterns have been noted. The basic molecular mechanisms responsible for maintaining the silenced state are quite well understood, as outlined in this collection. Consequently, we also know that epigenetic silencing has profound implications for cancer prevention, detection, and therapies. We now have drugs approved by the U.S. Food and Drug Administration (FDA) that are used to reverse epigenetic changes and restore gene activity to cancer cells. Also, because changes in DNA methylation can be detected with a high degree of sensitivity, many strategies are able to detect cancer early by finding changes in DNA methylation. The translational opportunities for epigenetics in human cancer research, detection, prevention, and treatment are, therefore, quite extraordinary.

Probable Questions:

1. What is nuclear transplantation. Briefly explain.
2. Describe the procedure of nuclear transplantation.
3. How epigenetics affects aging?
4. How epigenetics affects immunity in human?
5. How epigenetics affects Neuropsychiatric disorders
6. Discuss pediatric syndromes and epigenetics.
7. Discus role of epigenetics in cancer formation.
8. What do you know about epigenetic determinants of cancer ?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

ELECTIVE THEORY PAPER (ZET -403)

CYTOGENETICS AND MOLECULAR BIOLOGY

Unit II - Transposons and Extra-nuclear inheritance -

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 404 (CYTOGENETICS AND MOLECULAR BIOLOGY)	VII	**Mobile genetic elements: Characteristics of transposable elements in prokaryotes and eukaryotes; AC/DS system in maize; P element in *Drosophila*; Salmonella phase variation; retrosposons**	0.5	1	1	
	VIII	**Extra-nuclear inheritance: Streptomycin resistance in *Chlamydomonus*; Kappa particles; criteria for extra-chromosomal inheritance, infectious heredity.**		1	1	

Unit-VII

Mobile genetic elements: Characteristics of transposable elements in prokaryotes and eukaryotes; AC/DS system in maize; P element in *Drosophila*; Salmonella phase variation; retrosposons

Objective: In this unit we will discuss about different types of transposable genetic elements both in prokaryotes and eukaryotes. We will also discuss salmonella phase variation and retroposons.

Definition of Transposons:

Presence of transposable elements was first predicted by Barbara McClintock in maize (corn) in late 1940s. After several careful studies, she found that certain genetic elements were moving from one site to an entirely different site in the chromosome. She called this phenomenon of changing sites of genetic elements as transposition and those genetic elements were called by her as controlling elements. These controlling elements were later on called as transposable elements by Alexander Brink. In late 1960s this phenomenon was also discovered in bacteria. Consequently, the molecular biologists called them as Transposons. A transposon may be defined as: **"**a DNA sequence that is able to move or insert itself at a new location in the genome." The phenomenon of movement of a transposon to a new site in the genome is referred to as transposition.

Transposons are found to encode a special protein named as transposase which catalyses the process of transposition. Transposons are particular to different groups of organisms. They constitute a fairly accountable fraction of genome of organisms like fungi, bacteria, plants, animals and humans. Transposons have had a major impact on changing or altering the genetic composition of organisms. Transposons or transposable genetic elements are often referred to as 'mobile genetic elements' also. They can be categorized on different bases like their mode of transposition or on the basis of the organisms in which they are present.

Types of Transposons:

Different transposons may change their sites by following different transposition mechanisms.

On the basis of their transposition mechanism, transposons may be categorized into following types:

(i) Cut-and-Paste Transposons:

They transpose by excision (cutting) of the transposable sequence from one position in the genome and its insertion (pasting) to another position within the genome (Fig. 1).

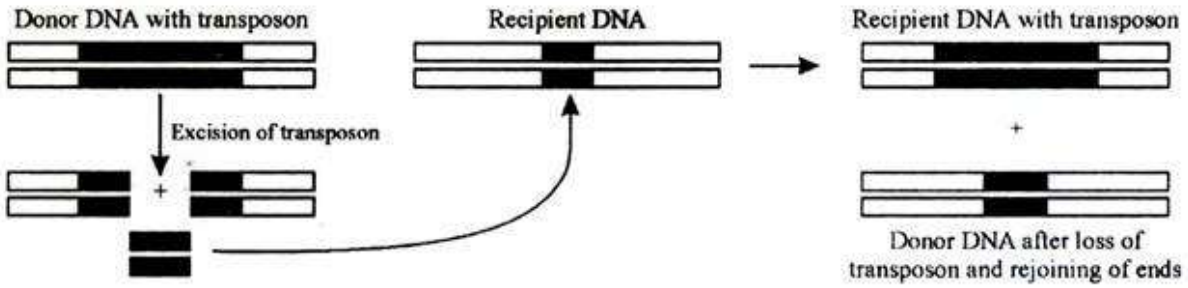


Fig. 1. Cut and Paste Transposons.

The cut-and-paste transposition involves two transposase subunits. Each transposase submit binds to the specific sequences at the two ends of transposon. These subunits of transposase protein then come together and lead to the excision of transposon.

This excised 'transposon-Transposase Complex' then gets integrated to the target recipient site. In this manner, the transposon is cut from one site and then pasted on other site by a mechanism mediated by transposase protein (Fig. 2).

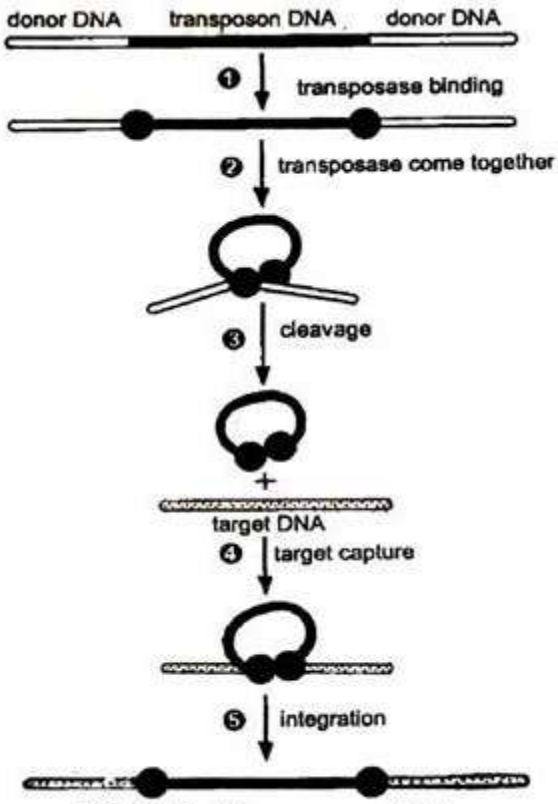


Fig. 2. Role of Transposase protein in cut-and-paste transposition.

Examples of cut-and-paste type of transposons are IS-elements, P-elements in maize, hobo-elements in Drosophila etc.

(ii) Replicative Transposons:
They transpose by a mechanism which involves replication of transposable sequence and this copy of DNA, so formed, is inserted into the target site while the donor site remains unchanged (Fig. 3). Thus, in this type of transposition, there is a gain of one copy of transposon and both-the donor and the recipient DNA molecule are having one-one transposable sequence each, after transposition.

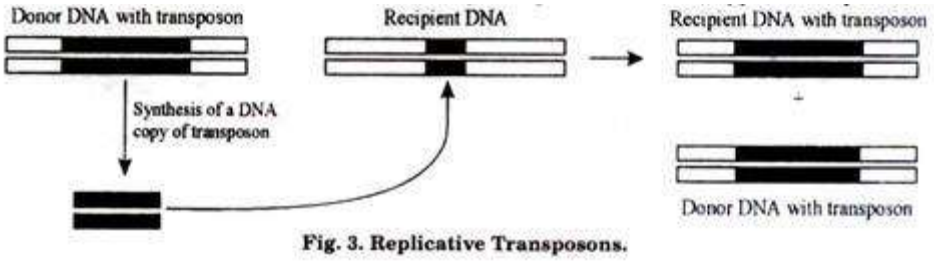


Fig. 3. Replicative Transposons.

Figure: Tn3-elements found in bacteria are good examples of such type of transposons.

(iii) Retro Elements:
Their transposition is accomplished through a process which involves the synthesis of DNA by reverse transcription (i.e. RNA DNA) by using elements RNA as the template (Fig. 4). This type of transposition involves an RNA intermediate, the transposable DNA is transcribed to produce an RNA molecule.

This RNA is then used as a template for producing a complementary DNA by the activity of enzyme reverse transcriptase. This single stranded DNA copy so formed, is then made double stranded and then inserted into the target DNA site. The transposable elements which require reverse transcriptase tor their movement are called retro transposons.

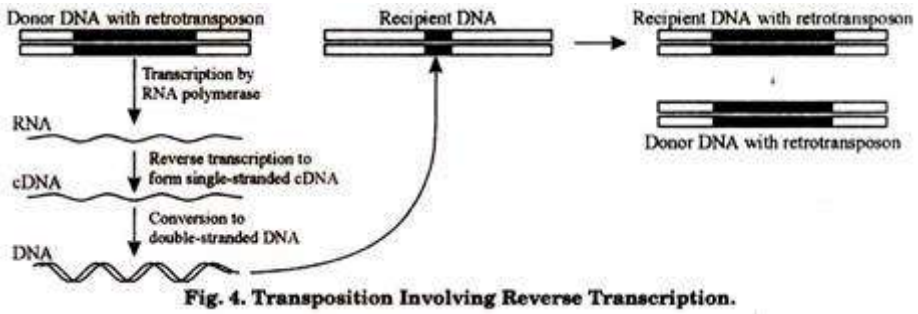


Fig. 4. Transposition Involving Reverse Transcription.

The Retro elements may be viral or non-viral. Out of these two, the non-viral retro elements are important and may further be classified as:

(A) Retrovirus like elements:
They carry long terminal repeats (LTR). Examples are copia, gypsy elements in Drosophila.

Retroposons:
LTR are absent. Examples are LINEs and SINEs in humans.

Structure of a Transposon:

Transposons are stretches of DNA that have repeated DNA segments at either end. A transposon consists of a central sequence that has transposes gene and additional genes. This is flanked on both sides by short repeated DNA segments. The repeated segments may be direct repeats or inverted repeats. These terminal repeats help in identifying transposons.

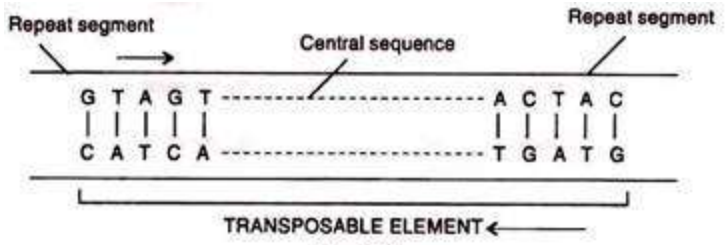


Fig. 18.2.

The number of repeated nucleotides is uneven 5 or 7 or 9 nucleotides are due to its method of insertion at the target site.

Target Sequence:

The site where a transposon is inserted is called target site or recipient site. Before the transposon is moved into the target site, the target sequence is duplicated. The two copies formed move apart. The transposon is inserted in between the two copies of the target sequences.

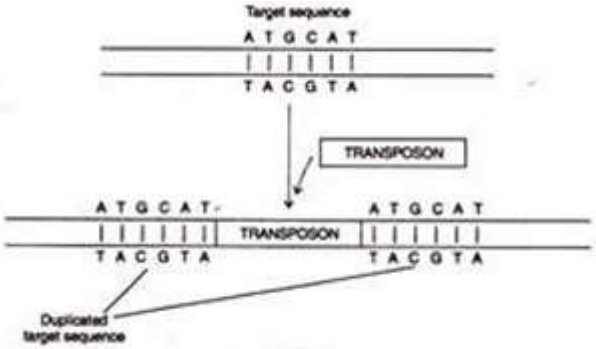


Fig. 18.3.

Mechanism of Transposition:

The enzyme transposase present in the transposon itself makes nicks or cuts in each strand of the target sequence. The target sequence is duplicated and two copies move away to make way for the transposon in the centre. The transposon then fixes itself into the two free ends generated. The nicks are sealed by ligase and two strands become continuous.

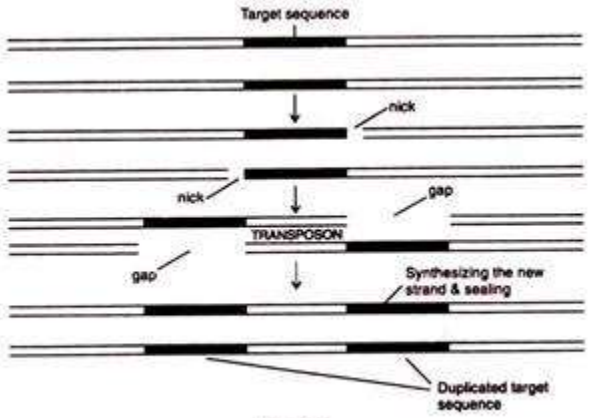


Fig. 18.4.

Transposable genetic elements in Prokaryotes:

The four transposable genetic elements in prokaryotes are: (1) Bacterial Insertion Sequences (2) Prokaryotic Transposons (3) Insertion-Sequence Elements and Transposons in Plasmids and (4) Phage mu.

(1) Bacterial Insertion Sequences:

Insertion Sequences or Insertion-Sequence (IS) Elements:

Insertion sequences, or insertion-sequence (IS) elements, are now known to be segments of bacterial DNA that can move from one position on a chromosome to a different position on the same chromosome or on a different chromosome. An IS element contains only genes required for mobilizing the element and inserting the element into a chromosome at a new location. Is elements are normal constituents of bacterial chromosome and plasmids. When IS elements appear in the middle of genes, they interrupt the coding sequence and inactivate the expression of that gene. Owing to their size and in some cases the presence of transcription and translation termination signals, IS elements can also block the expression of other genes in the same operon if those genes are downstream from the promoter of the operon. IS elements were first found in E. coli as a result of their affects on the expression of a set of three genes whose products are needed to metabolize the sugar galactose as a carbon source. Careful investigations showed that the mutant phenotypes resulted from the insertion of an approximately 800 base pairs (bp) DNA segment into a gene. This particular DNA segment is now called insertion sequencel (IS1).

Properties of IS Elements:

Is1 is the genetic element capable of moving around the genome. It integrates into the chromosome at locations with which it has no homology, thereby distinguishing it from recombination. This event is an example of transposition event. There are number of IS elements that have been identified in E. coli, including IS1, IS2, and IS 10, each present in 0 to 30 copies per genome, and each with a characteristic length and unique nucleotide sequence.

IS 1 is 768 bp long, and is present in 4 to 19 copies on the E. coli chromosomes. IS2 is present in 0 to 12 copies on the E. coli chromosome and in one copy on the F plasmid, and IS 10 is found in a class of plasmids called R plasmid that can replicate in E. coli (Fig. 12.1).

Among prokaryotes, the IS elements are normal cell constituents, that is, they are found in most cells. Altogether, IS elements constitute approx. 0.3% of the cell's genome. All IS elements that have been sequenced, end with perfect or nearly perfect inverted terminal repeats (IRs) of between 9 and 41 bp. This means that essentially the same sequence is found at each end of an IS but in opposite orientations.

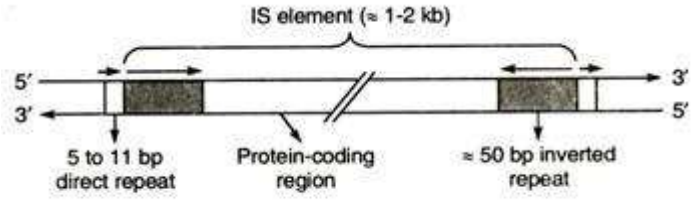


Fig. 12.1. Structure of bacterial IS elements.

IS Transposition:

When transposition of an IS element takes place, a copy of the IS element inserts into a new chromosome location while the original IS elements remains in place. That is, transposition requires the precise replication of the original IS element, using the replication enzymes of the host cell. The actual transposition also requires an enzyme encoded by the Is element called transposase.

The IR sequences are essential for the transposition process, that is, those sequences are recognized by transposase to initiate transposition. Is elements insert into the chromosomes at sites with which they have no sequence homology?

Genetic recombination between non-homologous sequences is called illegitimate recombination. The sites into which IS elements insert are called target sites. The process of IS insertion into a chromosome is shown in Figure 12.2. Firstly, a staggered cut is made in the target site and the IS element is then inserted, becoming joined to the jingle-stranded ends.

The gaps are filled in by DNA polymerase and DNA ligase, producing an integrated IS element with two direct repeats of the target site sequence flanking the IS element. 'Direct' in this case means that the two sequences are repeated in the same orientation. The direct repeats are called target site duplications. The sizes of target site duplication vary with the IS elements, but tend to be small. Integration of some IS elements show preference for certain regions, while others integrate only at particular sequences.

All copies of a given IS element have the same sequence, including that of the inverted terminal repeats. Mutations that affect the inverted terminal repeat sequence of IS elements affect transposition, indicating that the inverted terminal repeat sequences are the key sequences recognized by transposase during a transposition event.

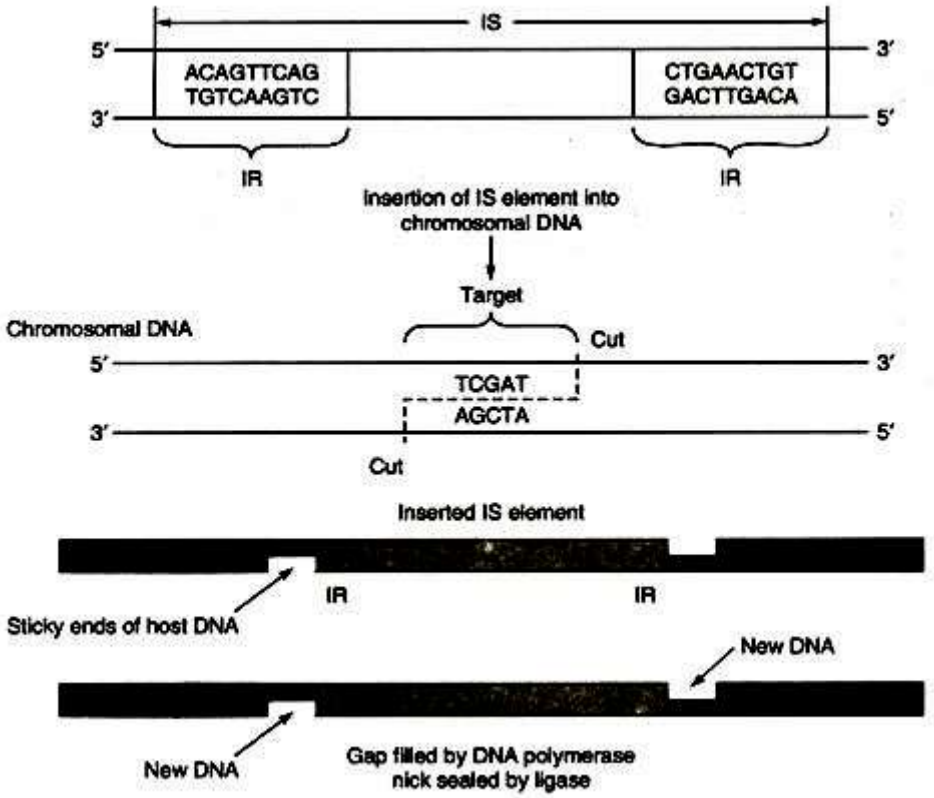


Fig. 12.2. Schematic presentation of the integration of an IS elements into chromosomal DNA.

(2) Prokaryotic Transposons:

A transposon (Tn) is more complex than an IS elements. A transposon is a mobile DNA segment that, like an IS element, contains genes for the insertion of the DNA segment into the chromosome and for the mobilization of the element to other locations on the chromosome. There are two types of prokaryotic transposons: composite transposons and non-composite transposons.

(a) Composite Transposons:

They are complex transposons with a central region containing genes, e.g., drug resistance genes, flanked on both sides by IS elements (also called IS modules). Composite transposons may be thousands of base pairs long. The IS elements are both of the same types and are called IS-L (for "left") and IS-R (for "right"). Depending upon the transposon, IS-L and IS-R may be in the same or inverted orientation relative to each other. Because the ISs themselves have terminal inverted repeats, the composite transposons also have terminal inverted repeats.

Figure 12.3 shows the structure of the composite transposon Tn 10 to illustrate the general features of such transposons. The Tn 10 transposon is 9,300 bp long and consists of 6,500 bp of central, nonrepeating DNA containing the tetracycline resistance gene flanked at each end with a 1,400-bp IS element. These IS elements are designated IS10L and IS10R and are arranged in an inverted orientation. Cells containing Tn 10 are resistant to tetracycline resistance gene contained within the central DNA sequence.

Transposition of composite transposon occurs because of the function of the IS elements they contain. One or both IS element supplies the transposase. The inverted repeats of the IS elements at the two ends of the transposon are recognized by transposase to initiate transposition (as with transposition of IS elements).

Transposition of Tn 10 is rare, occurring once in 10 cell generations. This is the case because less than one transposase molecule per cell generation is made by Tn 10. Like IS elements, composite transposons produce target site duplications after transposition.

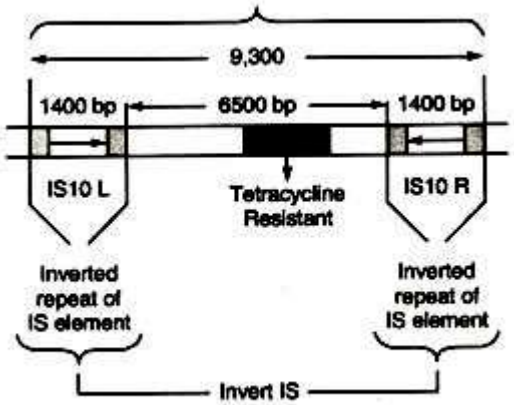


Fig. 12.3. Detailed structure of Tn10 transposon.

(b) Non-composite Transposons:

They like composite transposons, contain genes such as those for drug resistance. Unlike composite transposons, they do not terminate with IS elements. However, they do have the repeated sequences at their ends that are required for transposition. Tn3 is a non-composite transposon.

Tn3 has 38 bp inverted terminal repeats and contains three genes in its central region. One of those genes, bla, encodes β-lactamase which breaks down ampicillin and therefore makes cells containing Tn3 resistant to ampicillin. The other two genes, tnpA and tnpB, encode the enzymes transposase and resolvase that are needed for transposition of Tn3 (Fig. 12.4). Transposase catalyzes insertion of the Tn into new sites, and resolvase is an enzyme involved in the particular re-combinational events associated with transposition.

Resolvase is not found in all transposons. The genes for transposition are in the central region for non-composite transposons, while they are in the terminal IS elements for composite transposons. Non composite transposons also cause target site duplications when they move.

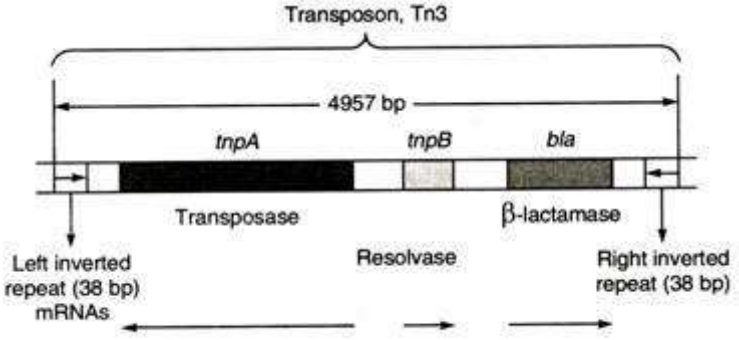


Fig. 12.4. Detailed structure of Tn3 transposon.

(c) Mechanism of Transposition in Prokaryotes:

Several different mechanisms of transposition are employed by prokaryotic transposable elements. And, as we shall see later, eukaryotic elements exhibit still additional mechanisms of transposition. In E. coli, we can identify replicative and conservative (non-replicative) modes of transposition. In the replicative pathway, a new copy of the transposable element is generated in the transposition event. The results of the transposition are that one copy appears at the new site and one copy remains at the old site. In the conservative pathway, there is no replication. Instead, the element is excised from the chromosome or plasmid and is integrated into the new site (Fig. 12.5).

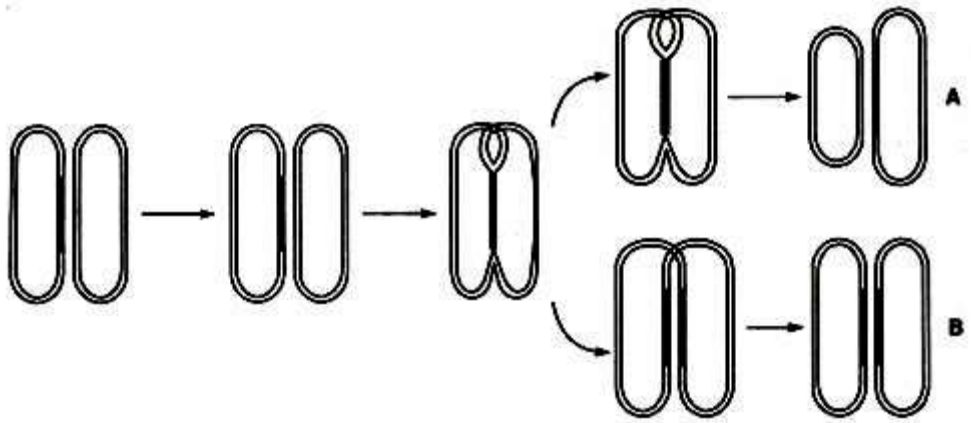


Fig.12.5. A. Conservative mode of transposition B. Replicative mode of transposition.

Replicative Transposition:

The transposition of Tn3 occurs in two stages. Firstly, the transposase mediates the fusion of two molecules, forming a structure called a cointegrate. During this process, the transposon is replicated, and one copy is inserted at each junction in the cointegrate. The two Tn3 are oriented in the same direction. In the second stage of transposition, the tnpR-encoded resolvase mediates a site-specific recombination event between the two Tn3 elements. This event occurs at a sequence in Tn3 called res, the resolution site, and generates two molecules, each with a copy of the transposon.

The tnpR gene-product also has another function, namely, to repress the synthesis of both the transposase and resolvase proteins. This repression occurs because the res site is located in between the tnpA and tnpR genes. By binding to this site, the tnpR protein interferes with the synthesis of both gene-products, leaving them in chronic short supply. Consequently, the Tn3 element tends to remain immobile (Fig. 12.6).

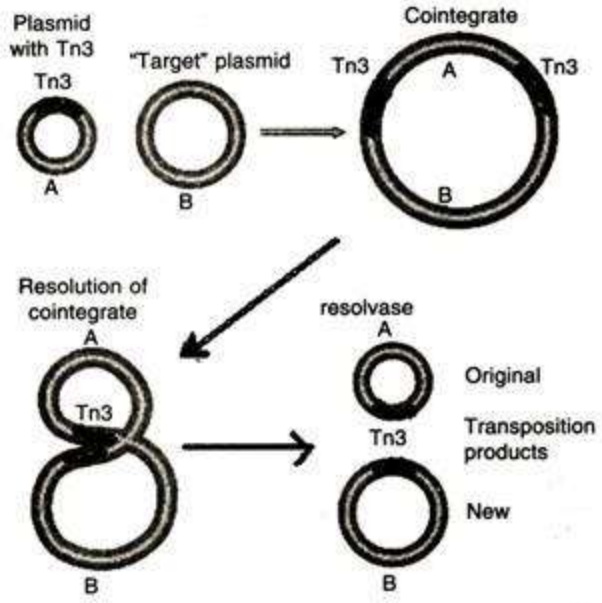


Fig. 12.6. Process of Tn3 transposition through cointegrate intermediate.

Conservative transposition:

Some transposons, such as Tn10, excise from the chromosome and integrate into the target DNA. In these cases, DNA replication of the element does not occur, and the element is lost from the site of the original chromosome. This mechanism is called conservative (non replicative) transposition or simple insertion. Tn 10, e.g., transposes by conservative transposition.

Insertion of a transposon into the reading frame of a gene will disrupt it, causing a loss of function of that gene. Insertion into gene's controlling region can cause changes in the level of expression of the gene. Deletion and insertion events also occur as a result of activities of the transposons, and from crossing-over between duplicated transposons in the genome.

(3) IS Elements and Transposons in Plasmids:

The transfer of genetic material between conjugating E. coli is the result of the function of the fertility factor F. The F factor, a circular double stranded DNA molecule, is one of the example of bacterial plasmid. Plasmids such as F that are also capable of integrating into the bacterial chromosomes are called episomes. F factor consists of 94,500 bp of DNA that code for a variety of proteins.

The important elements are:

(i) Transfer gene (tra) required for the conjugation transfer of the DNA.

(ii) Genes that encode proteins required for the plasmid's replication,

(iii) Four IS elements, two copies of IS3, one of IS2, and one of an insertion sequence element called gamma- delta.

It is because the E. coli chromosome has copies of these four insertion sequence at various positions that the F factor can integrate into the E. coli chromosome at different sites and in different orientations with homologous sequence of the insertion elements.

Another class of plasmids that has medical significance is the R plasmid group, which was discovered in Japan in the 1950s, during the cure for dysentery. The disease is the result of infection by the pathogenic bacterium Shigella. Shigella was found to be resistant to most of the commonly used antibiotics.

Subsequently, they found that the genes responsible for the drug resistances were carried on R plasmids, which can promote the transfer of genes between bacteria by conjugation, just as the F factor. One segment of an R plasmid that is homologous to a segment in the F factor is the part needed for the conjugal transfer of genes. That segment and the plasmid-specific genes for DNA replication constitute what is called the RTF (resistance transfer factor) region (Fig. 12.7). The rest of the R plasmid differs from type to type and includes the antibiotic-resistance genes or other types of genes of medical significance, such as resistance to heavy metal ions.

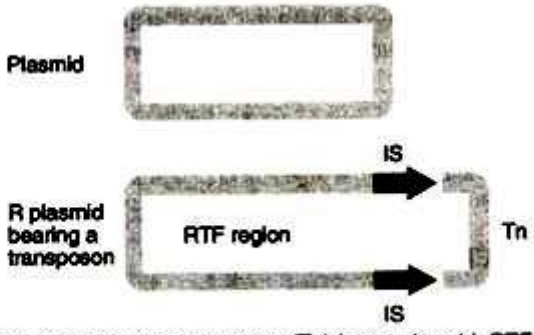


Fig. 12.7. The insertion of a transposon (Tn) into a plasmid. RTF represents the resistance-transfer functional genes of the plasmid.

The resistance genes in R plasmid are, in fact, transposons, that is each resistance gene is located between flanking, directly repeated segments such as one of the IS modules (Fig. 12.8). Thus, each transposon with its resistance gene in the R plasmid can be inserted into new location on other plasmids or on the bacterial chromosome, while at the same time leaving behind a copy of itself in the original position.

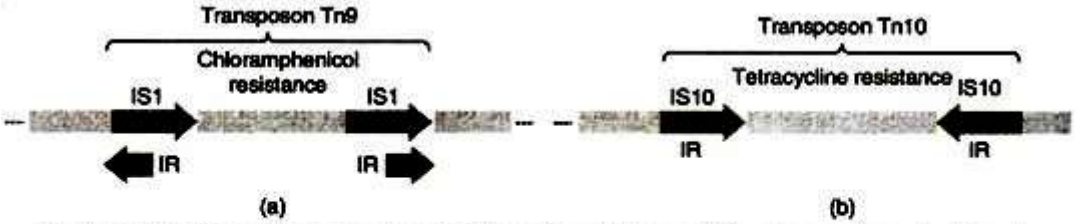


Fig. 12.8. Two different transposons having different inverted repeat (IR) regions and carrying different drug-resistance genes. (a) Tn9 has a short IR region, because the two IS1 elements are in the same orientation and each element has a short inverted repeat. (b) Tn10 has a large IR region because the two IS10 components have opposite orientations, and the entire IS10 sequence constitutes the inverted repeat.

(4) Phage mu:

Phage mu is a normal-appearing phage. We consider it here because, although it is a true virus, it has many features in common with IS elements. The DNA double helix of this phage is 36,000 nucleotides long-much larger than an IS element. However, it does appear to be able to insert itself anywhere in a bacterial or plasmid genome in either orientation. Once inserted, it causes mutation at the locus of insertion-again like an IS element. (The phage was named for this ability: mu stands for "mutator.")

Normally, these mutations cannot be reverted, but reversion can be produced by certain kinds of genetic manipulation. When this reversion is produced, the phages that can be recovered showing no deletion, proving that excision is exact and that the insertion of the phage therefore does not involve any loss of phage material either. Each mature phage particle has on each end a piece of flanking DNA from its previous host (Fig. 12.9). However, this DNA is not inserted anew into the next host. Its function is unclear. Phage mu also has an IR sequence, but neither of the repeated elements is at a terminus.

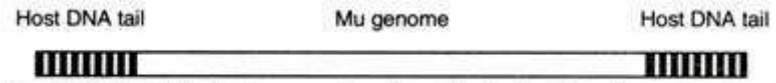


Fig. 12.9. The DNA of a free mu phage has tails derived from its previous host.

Mu can also act like a genetic snap fastener, mobilizing any kind of DNA and transposing it anywhere in a genome. For example, it can mobilize another phage (such as λ) or the F factor. In such situations, the inserted DNA is flanked by two mu genomes (Fig. 12.10).

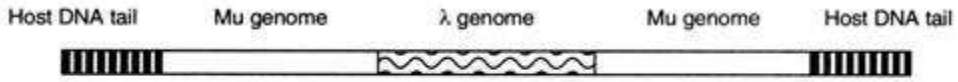


Fig. 12.10. Phage mu can mediate the insertion of phage λ into a bacterial chromosome, resulting in a structure like the one shown here.

Transposable elements in the eukaryotic organisms:

Transposons have been discovered in eukaryotic organisms also, e.g., controlling elements in maize, Tam1 elements in Antirrhinum majus, Ty elements in Yeast and FB elements in Drosophila. These elements can be divided into two main classes.

1. This class includes the transposable elements that are similar to those found in bacteria. These elements contain inverted repeats at their ends and generate short direct repeats of the target DNA at the sites of their insertion. These elements are always located in the host genome and cannot survive outside the genome. Controlling elements in maize and P elements in Drosophila belong to this class of transposable elements.

2. Retroposons (Retro transposons). Retroposons are DNA elements formed by the reverse transcription of retroviruses. This class of retroviruses and other sequences are transposed via RNA. Transposition of retroposons occurs through RNA intermediates.

Controlling Elements in Maize:

In 1940's Barbara McClintock discovered changes in maize genome during somatic cell division. The changes were genetically controlled aberrations, such as, deficiencies, duplications, inversions, translocations and ring chromosomes. These changes were found to be caused by a genetic system named Dissociation-Activator (Ds-Ac) system.

McClintock termed these genetic elements Ds and Ac as controlling elements in 1956. Since then, several systems of controlling elements have been discovered in maize. These elements are classified into two groups: autonomous and non-autonomous.

1. Autonomous elements:

The controlling elements which have the ability of their own excision and transposition are called autonomous elements, e.g., Activator (Ac), Suppressor mutator (Spm) and Enhancer (En).

2. Non-autonomous elements:

These elements do not have the ability of transposition. Non-autonomous elements have originated from autonomous elements through the loss of transacting functions which are required for transposition. A single type of autonomous element and different non-autonomous elements derived from it form a family.

Deletions of different lengths and different regions from an autonomous element give rise to different types of non-autonomous elements. Such elements change their position in response to an autonomous element of the same family present in the genome.

Non-autonomous element is activated in trans by its related autonomous element. Examples of non-autonomous elements are Dissociation (Ds), defective suppressor mutator (dSpm), and Inhibitor (I).

Dissociation-Activator (DS-Ac) System:

The main features of the Ds-Ac system in maize are as follows:

(i) An Ac element can exist in a number of states similar to other genes, and it controls the activity and time of action of the Ds element.

(ii) Ac and Ds, both exhibit inter-chromosomal as well as intra-chromosomal movements (transposition). The movement occurs through excision of these elements from one site and their insertion at a new site.

(iii) Ds element is unstable in the presence of the Ac element in the same nucleus. When both the elements are present, loss (deletion) of a part of the chromosome 9 occurs if the chromosome 9 carries the Ds element. The deletion is caused by breakage of the chromosome at the site of Ds.

(iv) The genes lying adjacent to the Ds become inactivated.

(v) The number of Ac elements present in a genome has a negative relationship with the time of Ds action during the development. Therefore, the presence of Ac in a greater number delays the transposition of Ds during the development. This can be well explained in the maize endosperm which is triploid.

In maize endosperm, the number of Ac element may vary from 0 to 6. The dominant allel I at the C locus on chromosome 9 inhibits colour formation in the aleurones of kernels so that the kernels having the I allele are colourless. In the presence of recessive allele i, colour develops normally in the aleurone. Therefore, an ii secondary nucleus fertilized by a pollen carrying i allele will produce coloured aleurone iii. But an ii secondary nucleus fertilized by a pollen carrying the dominant allele I will produce colourless endosperm Iii.

If both, Ac and Ds are present in the above I pollen, and Ds occupies a place within or near the C locus, coloured spots would be observed in many kernels. The coloured spots develop due to the transposition of the Ds element from the allele I during the stages of seed development which permits the c locus to produce aleurone colour. An increase in the number of Ac elements delays the dissociation of Ds. Thus variegation pattern in the kernel will differ according to the number of Ac. (Table 5.3).

TABLE 5.3. Effect of number of *AC* elements on the time of *DS* dissociation leading to chromosome breakage and kernel colour in maize

Genotype of endosperm	Time of dissociation of DS	Degree of colour spotting in kernel
Iii - DS	No dissociation	Colourless kernel
Iii-DS AC	Early dissociation	Large colour spots
Iii- DS AC AC	Dissociation at the stage	Small colour spots
Iii – DS AC AC AC	Dissociation at very late stage	Very few tiny colour spots

Organization of Ac and Ds elements:

Activator (Ac):

Activator (Ac) element is 4563 bp long and is autonomous in action. It has 11 bp inverted repeats at its both ends (Fig. 5.11). The target site for Ac insertion is 8 bp long; this target sequence is duplicated during the insertion as direct repeats. The Ac element has 5 exons (Fig. 5.11); transcription produces and mRNA of 35 00 bases which has a coding sequence for 807 codons. This element has two open reading frames.

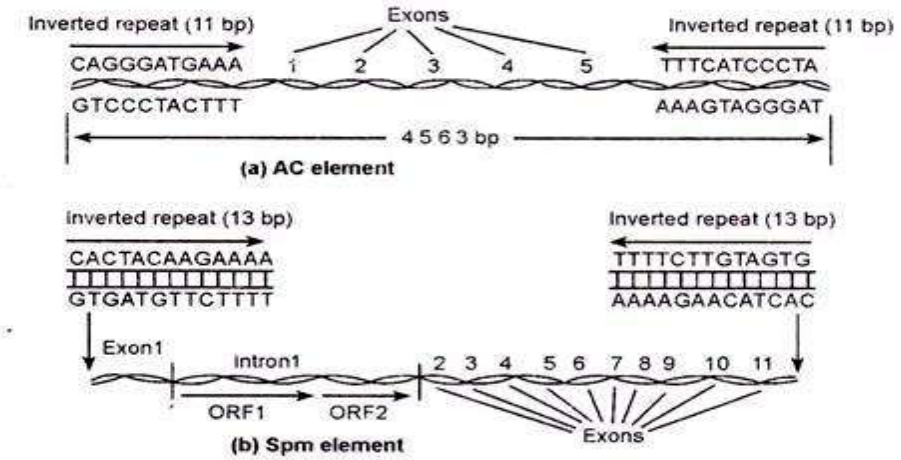


Fig. 5.11. Diagrammatic representation of *AC* and *Spm* elements in maize. Different *DS* elements are produced due to interstitial deletion in *AC*, while different *dSPm* elements are produced due to interstitial deletion in *Spm*.

Dissociation (Ds) element:

These elements are non-autonomous and are produced through interstitial deletions in the Ac element (Fig. 5.11). Based on the length and the region of deletion, Ds elements are grouped into several types as, Ds1, Ds2, Ds6, Ds9 Ds 2dl, and Ds2d2 etc. All the Ds elements contain the 11 bp inverted repeats at their ends. The Ds1 element represents an extreme case in that it has a large interstitial deletion so that only the terminal 11bp inverted repeats are retained.

Ds6 element possesses 1000 bp from each end of the Ac, the rest portion being deleted. Ds9 on the other hand, represents a very short deletion of about 194 bp. Further changes may also occur in the non-autonomous elements leaving them incapable of transposition, i.e., they become permanently stabilized.

Autonomous elements may also be subject to changes. During the different developmental periods of an individual these elements may undergo cycles of active and inactive phases; the phase changes are brought about by methylation of their DNA. A methylation in the target sequence $\frac{CAG}{GTC}$ of an element leads to a reversible inactivation of the element.

Effects of transposition of Ds:

Transposition of Ds causes breakage in the chromosome at the site from which the Ds element moves out. The mechanism of transposition is non-replicative. Following breakage, the acentric chromosome fragment is lost.

If the chromosome carrying Ds has dominant alleles, e.g. A, B, C and its homologue carries the recessive alleles a, b, c, the transposition of Ds will lead to breakage and loss of the fragment carrying the dominant alleles (Fig. 5 12.). In the progeny cells as a result only the recessive alleles a, b, c will be expressed.

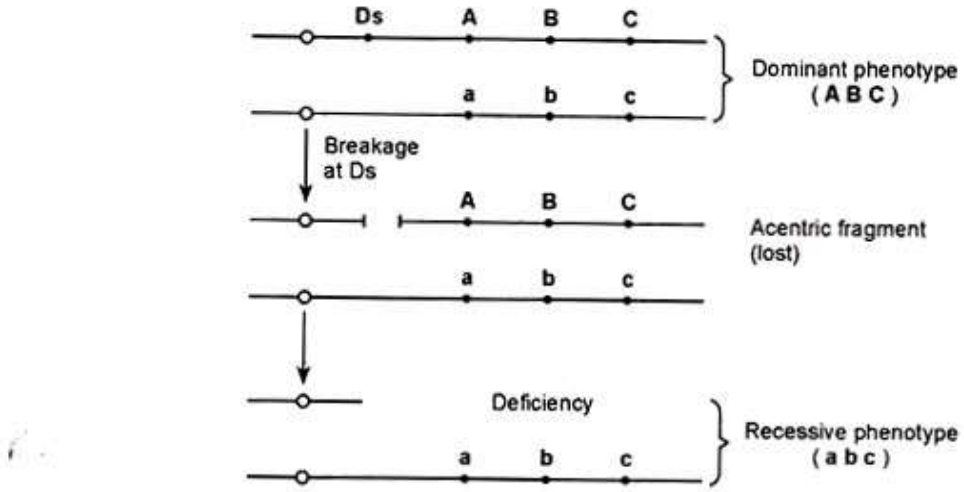


Fig. 5.12. Breakage at *DS* site produces an acentric fragment which is lost. As a consequence, the cell becomes deficient for genes *A*, *B* and *C* carried in the acentric fragment. Therefore, the expression of their recessive alleles present in the homologous chromosome is observable.

Alternatively, the broken ends of the two sister chromatids may join together as they are produced through chromosome replication. The acentric fragment, as a result, will form a U-

shaped structure which would be lost. The centric fragment, on the other hand, will form a dicentric chromatid bridge during anaphase. As a result of the tension created due to the centromere movement, the chromatid bridge will break at some point between them producing two dissimilar chromatids. In the next cycle, the broken chromatid ends will again fuse during chromosome replication.

This will, as earlier lead to the formation of dicentric chromatid bridges in both the daughter cells. Thus a chromatid-fusion-bridge-breakage cycle is generated (Fig. 5.13). In such a condition cells contain duplication for one or more genes along with deficiency for some other genes.

Duplication and deletion:

When the transposons are located on both the homologues but at different positions, pairing and recombination between them leads to the production of one deficient chromosome and one chromosome with duplication (Fig. 5.14).

Translocations caused by transposons:

Transposons located on non-homologous chromosomes can pair, and crossing over between them produces reciprocal translocations (Fig. 5.15).

Suppressor-Mutator (Spm) Elements:

Spm is nearly similar to the En (enhancer). It is a larger transposon than Ac and contains inverted repeats of 13bp at its ends. Its promoter is situated at the left and is responsible for the transcription of 8300bp of DNA. Spm is composed of two genes, tnpA and tnpB. The tnpA is a split gene containing 11 exons, the first intron being very long (Fig. 5.11). After splicing, a 2500 base mRNA is obtained which is translated into a protein of 621 amino acids. The first intron of the tnpA gene possesses two additional reading frames ORF l and ORF 2; both the reading frames are jointly called the tnpB gene. The proteins coded by tnpA and tnpB are required for several functions related to transposition. A deletion in the ORF 1 and ORF 2 regions produce defective Spm elements (dSpm).

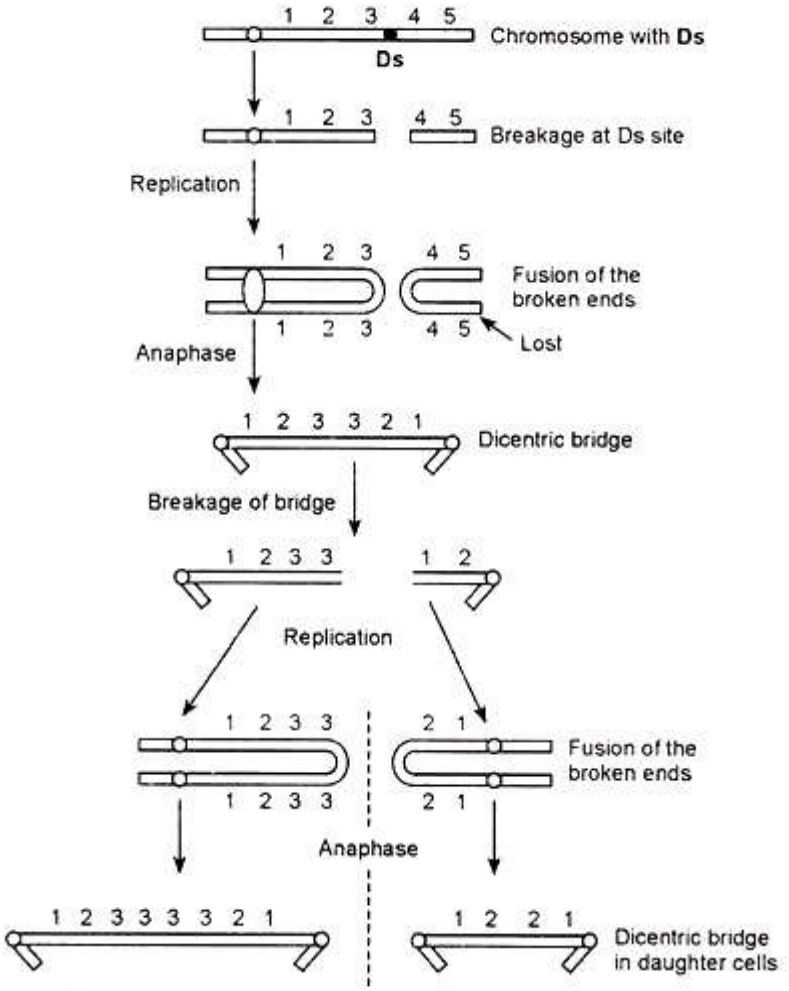


Fig. 5.13. Breakage-fusion-bridge cycle generated by chromosome breakage caused by the transposition of a *DS* element.

Transposition of the Spm into a gene completely inhibits the expression of the gene. But when dSpm insertion occurs within a gene the expression of the gene is reduced; such a gene is called dSpm- suppressive gene. Insertion of dSpm in the vicinity of a gene does not inhibit or reduce its expression but, on the other hand, enhances its expression. Therefore, such a gene is called "dSpm- dependent gene".

P Elements in Drosophila Melanogaster:

In Drosophila, certain strains when mated together produce hybrid dysgenesis (mutations, chromosome aberrations, distorted segregation at meiosis and sterility). In such crosses, the F, flies have normal somatic tissues but, their gonads do not develop.

A transposable genetic element known as "P element" or "P factor" has been found to be responsible for this condition. On the basis of the presence or the absence of the P factors, the flies are divided into two types.

I. P-type (Paternal Contributing):

Strains containing P elements in their chromosomes are designated as P-type. The number of P elements varies from 30 to 40 in the genome.

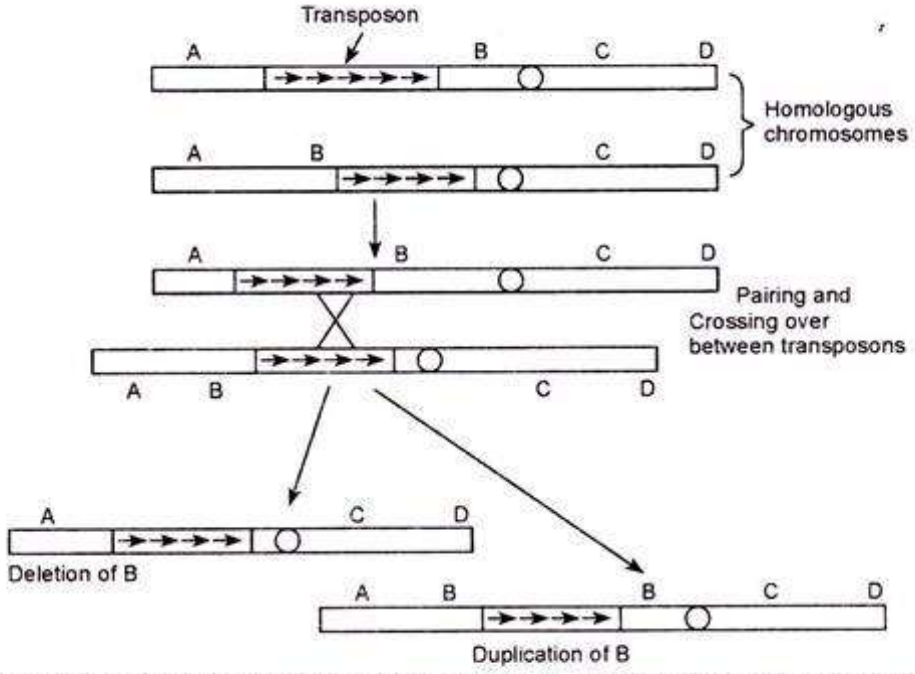


Fig. 5.14. Crossing over in the transposons located on homologous chromosomes produces deletion and duplication.

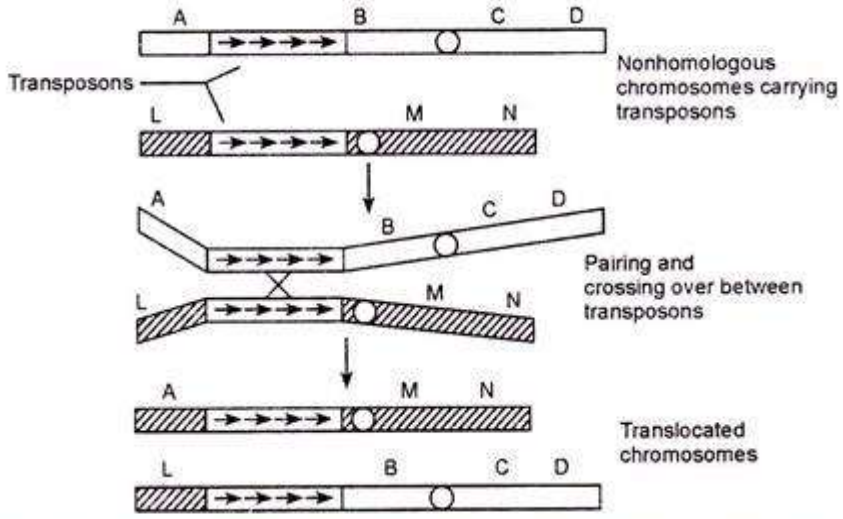


Fig. 5.15. Translocation caused by crossing over between the transposons located on non-homologous chromosomes.

P elements occupy several different positions on the chromosome. These factors are present on chromosomes as inactive component in the P-strain.

2. M-Type (Maternal Contributing):

M-strains do not contain P-elements in their chromosomes. Crosses involving P-Type male flies and M-type female flies produce F, flies showing hybrids dysgenesis. But when the female flies are P-type, the hybrids are normal irrespective of the male being P-type or M-type. (Fig. 5.16.).

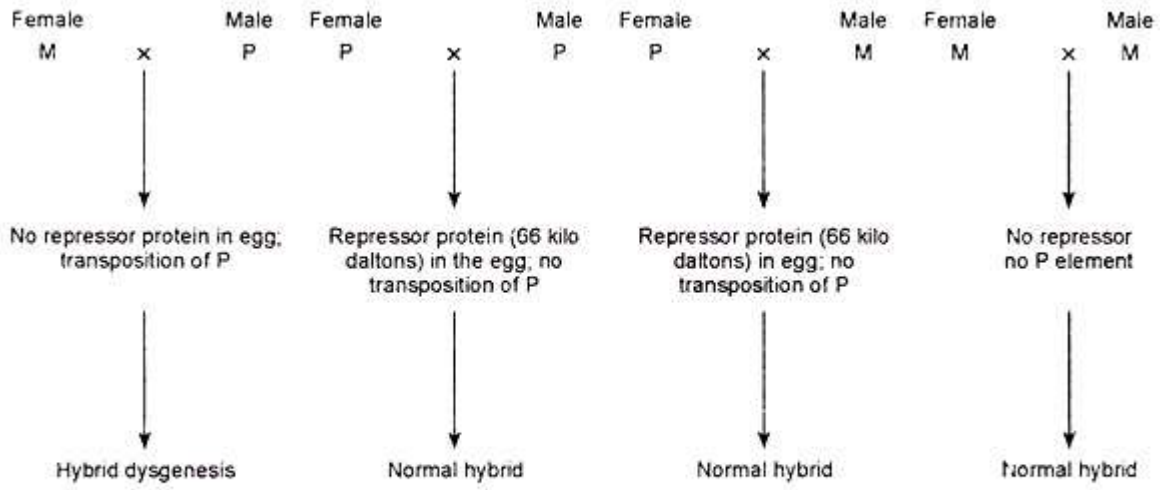


Fig. 5.16. Results of different types of P-M crosses in *Drosophila*. Hybrid dysgenesis occurs only when a P male is mated to an M-female. A 66 kilo dalton repressor protein is present in the egg cytoplasm of P-females : This repressor inhibits the transposition of P elements resulting into normal F_1 flies.

Organisation of P elements:

There are different types of the P element. The full size elements are about 3000 bp long and contain inverted repeats of 31 bp at their ends. Transposition of the P elements generates direct repeats of 8 bp on both sides of the site where transposition has taken place.

Interstitial deletions in P element produce different P elements of smaller sizes. Some of the small P element possess the gene coding for transposase while others do not have the complete gene. The latter type of P elements (which have an incomplete transposase gene) are activated by the transposase enzyme produced by another normal P element. The full size P element has 4 open reading frames designated as ORFO, ORF1, ORF2 and ORF3. The processing of the primary RNA transcript occurs by separate mechanisms in somatic and germinal tissues.

1. Processing in somatic tissues:

In somatic tissues, splicing occurs producing the mRNA which contains only three open reading frames, namely, ORFO, ORF1 and ORF2; the intron 3 is not spliced. Somatic cells contain a protein that binds to the intron 3, thus inhibiting the removal of this intron. This mRNA produces a 66,000 Dalton protein which functions as a repressor for transposition so that somatic tissues are not affected by P element.

2. Processing in the germ line:

In the germ line, the intron 3 binding protein is absent and therefore, all the introns are removed during processing of the primary transcript. The four reading frames ORFO, ORF1, ORF2 and

ORF3 are joined together to produce a large mRNA. This mRNA produces the 87,000 Dalton protein (the enzyme transposase) which leads to transposition.
Transposition occurs by the no-replicative mechanisms similar to that of Tn10 transposon. The enzyme transposase binds to 10 bp sequence adjacent to the 31 bp inverted repeats at ends of P. Transposition leaves a break at the original site of P and it produces gene mutation at the new (insertion) site of P. Both the events, therefore, generate adverse effects on the individual. The P line contains P-cytotype, while the M line contains M-cytotype. When chromosomes bearing P factors come into the M-cytotype, transposition occurs leading to hybrid dysgenesis. However, when chromosomes carrying the P elements come into the P cytoplasm, there is no transposition. This can be explained as follows (Fig. 5.16).
A repressor protein called 66,000 Dalton protein is present in the egg cytoplasm of flies containing P elements. However, the repressor protein is absent from the egg cytoplasm of M-females. When a P-male is crossed to an M-female, in the F_1 their P elements present in the paternal chromosome undergo transposition due to the absence of the repressor; this produces hybrid dysgenesis. But in crosses involving P-females, the transposition of P elements is prevented by the 66,000 Dalton repressor protein present in the egg cytoplasm; this yields normal fertile hybrids.

Retroposons (Retro-transposons):
Retroposons are transposable genetic elements which are mobilized through an RNA form. The DNA element is transcribed into RNA and then the RNA is copied by the enzyme reverse transcriptase into DNA which is inserted at a new site into the host genome. Retroposons include processed pseudo genes, small RNA pseudo genes (SnRNA) and Alu family in primates and rodents.
Some of the eukaryotic transposons are related to retroviral proviruses and mobilize through RNA intermediates. Retroposons differ from retroviruses in the sense that they do not pass through an independent infectious form. However, they do use the reverse transcription process to produce DNA. In order to understand retroposons, it is desirable to study the life cycle of a retrovirus.

Retroviruses:
They are infectious viruses containing single-stranded RNA (+ strand) which infect eukaryotic cells. Through reverse transcription, the viral RNA produces a complementary DNA (-) strand. The enzyme reverse transcriptase has exonuclease activity (RNAase H) by which it degrades the RNA strand from the RNA-DNA hybrid so produced. The same enzyme also synthesizes, by its polymerase activity, the complementary DNA strand (+ strand) to (-) strand.
This double-stranded DNA moves to the nucleus of the cell where one or more copies of it become integrated into the host genome; the enzyme involved in the insertion is integrase. This viral DNA genome integrated into eukaryotic host chromosome is called a provirus or retroposon. It remains as a endogenous provirus in the germ line. In other cells, the pro-viral DNA is transcribed to produce RNAs which function as (i) viral genome and (ii) mRNA to produce proteins that are structural components of the retrovirus.

In every viral particle, 2 copies of RNAs are packaged, making it a diploid virion. When two different retroviruses infect a single cell, the new viral particle may contain one chromosome from each of the two viruses; thus some virions may be heterozygous.

Organization of retroviral RNA and pro-viral DNA (Retroposon):

The retroviral RNA has direct repeats (R) varying from 10 to 80 nucleotides at its both ends (Fig. 5.17). An 80-100 base long unique region (U5) lies next to the R segment at the 5′-end. Similarly, at the 3′-end, left to the R segment, there occurs a unique region (U3) containing 170- 1260 nucleotides.

The coding region of the virus contains the genes gag (2000 bases), pol (2900 bases) and env (1800 bases). The reverse-transcribed DNA has a long terminal repeat (LTR) that s composed of the sequences "U3-R-U5" at both the ends (Fig. 5.17).

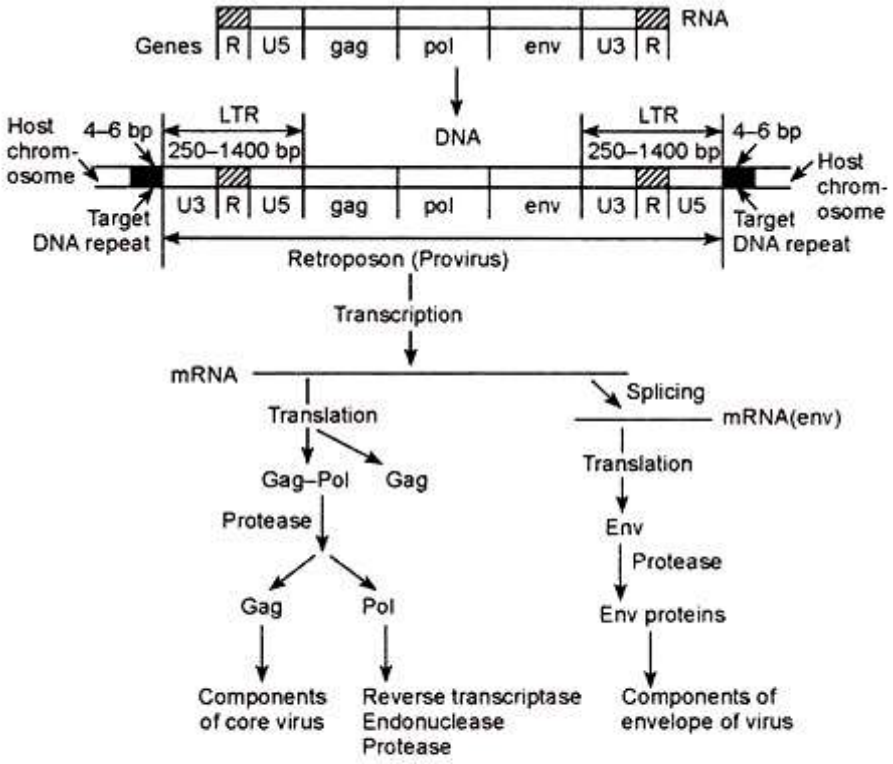


Fig. 5.17. Retroviral RNA, proviral DNA and the synthesis of polyproteins. Retroviral RNA has ends of short direct repeats. Genes are expressed as polyproteins that are processed into individual proteins. Number of bases in different regions of the retroviral RNA are 10-80 in R 170-1260 in U3, 80-100 in U5, ~2000 in *gag*, ~2900 in *pol* and ~1800 in ***env***.

Integration:

Integration of DNA into the host chromosome occurs through the linear form of DNA. Integration events are similar to those of transposable elements. The enzyme integrase makes staggered cuts at the target site which may be 4-6 bp in length. Direct repeats of the target DNA is produced during the integration. During this process, the U3 sequence loses 2 bp from left end and the U5 sequence loses 2 bp from the right end. A single cell receives up to 10 copies of a provirus.

Expression of viral genes:

The coding region of retroviruses consists of 3-4 genes, such as, gag pol, env sequences. Transcription of provirus produces the genomic RNA from which env mRNA is obtained by splicing (Fig. 5.17). Translation of the genomic RNA yields Gag, Gag-Pol and Env poly-proteins. Specific proteases cleave the poly-proteins into individual proteins through processing. After about 8 hour of infection, the poly-proteins together with viral genomic RNA begin to assemble under the plasma membrane. They attract the envelope proteins already present in the membrane. Nucleocapsid is formed by protein cleavages.

A segment of the host cell membrane is pinched off (like budding) and viral particle is released. During the process of infection, the viral particle becomes fused with the plasma membrane as a result of which, the RNA is released into the cell.

Transducing viral particles:

A retrovirus may carry genes from its host cell. This occurs when a deletion in the provirus (retroposon) occurs, thus fusing the viral and host genes. As shown in the Figure 5.18, the deletion of a part of the pro-viral DNA causes the transcription of DNA containing both, pro-viral DNA and the host DNA to produce a "fused mRNA."

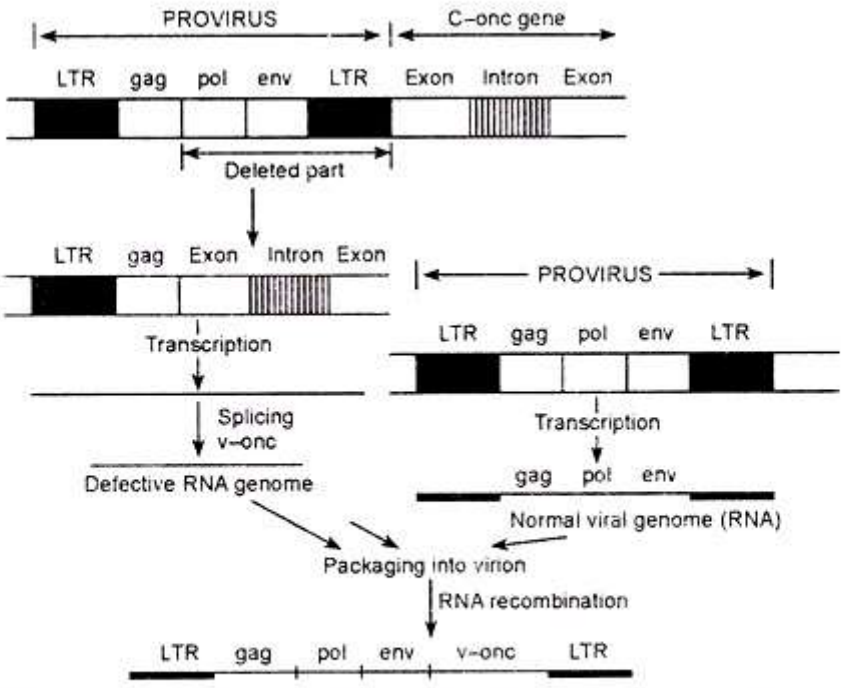


Fig. 5.18. Deletion in proviral DNA may produce fused mRNA containing some viral and cellular genes; this is defective viral RNA. A helper viral RNA produces the viral capsid and both the RNA genomes may become included in a single virion. Nonhomologous recombination will produce an RNA genome which contains both viral and host genes flanked by LTR sequences.

After splicing of the host mRNA the fused mRNA becomes shorter. In some cases, the c-onc gene of the host may be transcribed and fused with the viral mRNA. But this RNA is defective and cannot produce new virus. If the cell contains some normal provirus, it acts as a helper.

Some of the viral particles produced in such a way will contain one fused (defective) and one normal viral genome. Recombination between the two RNAs will produce an RNA genome that contains LTR along with the viral genes and host genes.
The c-onc gene is called v-onc gene when present in the viral genome. The properties of the host cell are drastically changed when it is infected by such a viral particle; it becomes a cancerous cell.

Retroposon-like Elements in Eukaryotes:
Retroposons or retro-transposon like elements are found in different eukaryotic organisms, such as, yeast, Drosophila, and mammals including human. These elements are classified into the following two groups.

I. Viral super family:
The retroposons that code for reverse transcriptase and integrase, and possess the ability of transposition belong to this family. They have long terminal repeats. Many retroposons also contain introns. They generate direct repeats of 4-6 bases in the target DNA. Examples of such elements are: Ty elements in yeast, copia in Drosophila and LINES LI in mammals.

II. Non-viral super family:
The retroposons belonging to this family do not code for proteins that have role in transposition. They are believed to have originated from RNA sequences through the process of reverse transcription; they do not contain either terminal repeats or introns. They generate direct repeats of target DNA containing 7-21 bp. Examples are SINES B1/Alu family in mammals, processed pseudo genes, transcript of RNA polymerase II.

Ty Elements in Yeast (Saccharomyces Cerevisiae):
Ty (Transposon yeast) elements are of divergent types and made a family of dispersed repetitive sequences on yeast genome. These elements are 63000 bases long and are grouped into two main classes, Ty1 and Ty917. A typical yeast genome contains about 30 copies of Ty] type and about 6 copies of Ty917 type elements. They have direct repeats of 350 bp at each end; these repeats are called delta (8).
A Ty element has two open reading frames TyA and TyB. The TyA protein represents the TyA reading frame, while the TyB protein represents the joint TyA and TyB regions. The TyA region codes for DNA-binding proteins, while TyB codes for reverse transcriptase, protease and integrase (like retrovirus). Ty elements are mobilized through an RNA intermediate and transposition is controlled by its genes. The element behaves like a retrovirus which has lost the coding region for the viral envelope.

Copia Elements in Drosophila:
The term copia denotes a large number of closely related sequences in Drosophila. The number of these retroposons per genome varies from 20 to 60. The copia element is 5146 bp long with terminal direct repeats of 276 bp and terminal inverted repeats of 13 bp.
It generates direct repeats of 5 bp in the target DNA at the site of insertion. Copia elements are dispersed and take different locations in different strains of Drosophila.

Sometimes copia elements are found as circular molecules of 5000 bp and 4700 bp in length. They contain a single reading frame of 4227 bp which shows homologous relationship with gag and pol sequences of retrovirus but the env sequence is absent. Therefore, copia cannot produce a virus particle.

Retroposon-Like Elements in Mammals:

In mammals, a large part of the repetitive DNA consists of retroposons. These are two main groups of these elements, called LINES and SINES. The LINES sequences are also called LI. They are long interspersed sequences dispersed in the genome. The average length of LINES is 6.5 kb. At their end, they contain sequences rich in adenine (A) nucleotides. LINES sequences are derived from the transcripts of RNA polymerase II. The number of copies of LINES ranges from 20,000 to 50,000 per mammalian genome.

Short interspersed sequences in mammalian genomes are called SINES. They are derived from the transcripts of RNA polymerase III. These elements do not posses a coding region, and are about 300 bp in length. Probably they originated from a transposition event like retroviruses and the RNA was copied into DNA by reverse transcriptase. The SINES family includes the "Alu- family". Alu family is a set of dispersed, related sequences (about 300 bp long) formed in human genome. Individual sequences have Alu cleavage sites at each end. There are about 300,000 (3×10^5) Alu sequences dispersed in the haploid human genome. The Alu sequences are flanked ' by short direct repeats indicating their resemblance to transposons.

Transposons in Humans:

Transposons in humans are in the form of repetitive DNA which consists of sequences that are interspersed within the entire human genome. These sequences are transposable and can move to different locations within the genome.

These are of following two types:

(1) SINEs (Short Interspersed Elements):

They are ~ 300 bp long and may be present about 5 lakh times in human genome. Alu sequences are the best characterized SINEs in humans.

These are termed as **'Alu'** elements because they contain specific nucleotide sequences which are cleaved by the restriction enzyme named Alul. Alu elements contain Direct Terminal Repeats (DTR) of 7-20 bp length. These DTRs help them in the insertion process during transposition.

(2) LINEs (Long Interspersed Elements):

They are ~ 6400 bp long and are present about 1 lakh times in the human genome. Most prominent example is LI sequence. These transposable elements are some of the most abundant and common families of moderately repeated sequences in human DNA.

Significance of Transposable Elements:

1. Transposons may change the structural and functional characteristics of genome by changing their position in the genome.
2. Transposable elements cause mutation by insertion, deletion, etc.

3. Transposons make positive contribution in evolution as they have tremendous impact on the alteration of genetic organisation of organisms.
4. They are useful as cloning vectors also, in gene cloning. For example, P-elements are frequently used as vector for introducing transgenes into Drosophila.
5. Transposons may also be used as genetic markers while mapping the genomes.
6. Transposon-mediated gene tagging is done for searching and isolation of a particular gene.

Probable Questions:

1. What is Transposable genetic element?
2. Describe types of transposons.
3. What is retrotransposons?
4. Describe the properties of IS elements.
5. What is composite transposons?
6. What is non composite transposons?
7. Describe the mechanism of transpositions?
8. Describe phage mu transposons.
9. What is Ac/Ds element? Describe its mechanism of transposition.
10. What is P elements in Drosophila.
11. Describe copia elements of Drosophila.
12. Describe Ty Elements in Yeast.
13. Discuss transposons present in human.
14. What are the significance of Transposable genetic elements?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.

Unit-VIII

Extra-nuclear inheritance: Streptomycin resistance in *Chlamydomonus*; Kappa particles; criteria for extra-chromosomal inheritance.

Objective: In this unit we will discuss about extra nuclear inheritance such as Streptomycin resistance in *Chlamydomonus* and also about kappa particles. We will also discuss about extra chromosomal inheritance.

Cytoplasmic Inheritance:

The existence of genes as segments of nucleic acid molecules, located in chromosome of nucleus, has been demonstrated by several experiments. The nuclear genes control the phenotypes of the organisms and are concerned with the transmission of hereditary character from one generation to next generation is known and predictable Mendelian fashion.

The inheritance of genes of nuclear chromosomes is characterised by the fact that the genes from male and female parents contribute equally to the genetic constitution of the offspring. Therefore, in it the reciprocal crosses between parents of different homozygous genotype will produce offspring's of identical phenotypes except for sex-linked genes. However, in certain cases, although male and female parents contribute equally their nuclear genes to the offspring's, the results show a non-Mendelian inheritance pattern and the result of reciprocal crosses varies.

These variations suggest that the genes for the inheritance of certain characters do not occur within the nucleus, but they are present ill cytoplasm and play an important role in transmission of certain specific traits, which are not controlled by nuclear genes. Therefore, it builds up the concept of cytoplasmic inheritance. The genes for cytoplasmic inheritance are independent, self-replicating nucleic acids.

Evidence for cytoplasmic inheritance was first reported by Correns in *Mirabilis jalapa* and by Bar in Pelargonium zonule in 1908. Rhoades described cytoplasmic male sterility in maize in 1933. In 1943, Sonneborn discovered kappa particles in *Paramoecium* and described its cytoplasmic inheritance. Presence of DNA in chloroplasts was first demonstrated by Ris in plant cell. In 1963, Nass and his co-workers proved the existence of DNA in mitochondria. Subsequently, from time to time, observations by several scientists have been reported the important role of cytoplasm in genetics. Thus, on the basis of observations made on cytoplasmic inheritance of some specific traits, it has been suggested that cytoplasm is also genetically active.

Terms and Definitions of Cytoplasmic Inheritance:

Extra-chromosomal inheritance, extra-nuclear inheritance, somal inheritance and maternal inheritance are all synonyms. All these terms can be defined as the inheritance of characteristics of only one of the two parents, usually the female parent to the progeny. The reciprocal crosses show consistent differences as well as there is a lack of segregation in F_2 and subsequent generations. The genes controlling cytoplasmic inheritance are present outside the nucleus and, in the cytoplasm, they are known as plasma genes, cytoplasmic genes, cytogeneses, extra nuclear genes or extra chromosomal genes. The sum total of the genes present in cytoplasm of

a cell is known as Plasmon. All the genes present in a plastid are known as plastoms. Similarly, all the genes present in a mitochondrion are known as chondrioms. The genes present in plastid and in mitochondrion are located in their own DNAs and are known as cp DNA and mtDNA, respectively. These DNAs are collectively termed organelle DNA.

Characteristics and Detection of Cytoplasmic Inheritance:
Cytoplasmic inheritances do not show Mendelian inheritance.

They show the following characteristic features:
i. Hereditary traits which are transmitted by cytoplasm do not show Mendelian segregation in crosses and in reciprocal crosses with respect to a particular set of characteristics controlled by a set of cytoplasmic genes produce dissimilar hybrids.

ii. Most of the recorded cytoplasmically inherited characteristics would follow the maternal line, i.e., uniparental mode of transmission. In higher plants and animals, ovum or egg cell is comparatively large and contains large amount of cytoplasm. But male gametes or sperms have very little amount of cytoplasm. So, under this situation, most of cytoplasmic factors are transmitted to the progeny through the ovum of mother.

It is known as maternal inheritance or trans-ovarian transmission. In this mode of transmission, all the offspring's of the parents have maternal condition and only female progeny can transmit the cytoplasmic characteristics to the succeeding generations. Hence the reciprocal crosses yield different or non-Mendelian results.

Characteristics of Mendelian Inheritance:
The inheritance pattern of characters of an organism as proposed by Mendel on the basis of monohybrid and di-hybrid crosses is referred to as Mendelian inheritance.

It shows the following characteristic features:
i. Contribution of both male and female is equal, hence results from reciprocal crosses are similar.
ii. Segregation produces the phenotypes ratio 3 : 1 and genotype ratio 1 : 2 : 1 in the F_2 generation of a monohybrid cross and a typical phenotype ratio 9 : 3 : 3 : 1 in di-hybrid crosses.
Mendelian inheritance pattern is regarded as a sufficient evidence for a gene to be located in chromosomes; such genes are called nuclear genes or simply as genes.

Maternal Inheritance*:*
Maternal inheritance means the inheritance controlled by extra-chromosomal, i.e., cytoplasmic, factors that are transmitted to the succeeding generation through the egg of female organism.

They show the following features:
i. reciprocal differences in F_1;
ii. which in most cases disappears in F_2;

iii. a smaller variation in F_2 as compared to that in F_3.

Maternal inheritance may be, broadly speaking, of two kinds:
i. If some treatments (chemical poison, heat shock etc.) are applied to the female parent, it may affect the egg's cytoplasm. As a result subsequent offspring's are modified in some way. Effects of this kind are called Dauer-modifications or persisting modifications.
It is observed that when protozoa are treated experimentally with chemical poisons or heat shocks, the treatments induce several morphological abnormalities in them. Such abnormalities go on decreasing generation after generation and, eventually, disappear completely through cell division if the treatments are removed. Further evidences also come from fruit flies subjected to heat treatment and from bacteria treated with chemicals.

ii. Other kinds of maternal inheritance are also known which do not depend upon the repeated application of an external stimulus to the cytoplasm. In this case, maternal inheritance is truly controlled by independent cytoplasmic genes. Maternal effects reflect the influence of the mother's gene on developing tissues. Many important characteristics of both animal and plants show maternal effects of which some examples axe described next.

Difference between Nuclear Traits and Extra-Nuclear Traits:

1. Reciprocal Differences:
Differences in the results of reciprocal crosses would suggest a deviation from the pattern of Mendelian autosomal gene transmission. According to Mendelian inheritance, the chromosome complement in male and female gametes obtained from the same species would be similar; reciprocal crosses should give same results (♀A x♂ B =♀B x ♀'A). The only exception to this expectation is sex- linked inheritance which can be explained on the basis of transmission of sex chromosomes.
If sex linkage is ruled out, differences in the result of reciprocal crosses would indicate that one parent (maternal) is exerting a greater influence than the other on a particular trait. This is because cytoplasm does not divide in a precise manner like the chromosomes during the process of cell division during gametogenesis. Female gametes usually contribute more cytoplasm to the zygote.
Consequently for characters having cytoplasmic control, differences in reciprocal crosses are observed. As shown in the Fig. 10.1, if two strains A and B respectively having genotypes AA and BB and cytoplasm's a and b are crossed reciprocally, we will get two hybrids AB (a) and AB (b) [cytoplasm is indicated in parentheses]. In case of maternal inheritance, AB (a) and AB (b), despite having same nuclear genotype, will differ. AB (a) will resemble strain A or AA (a) and AB (b) will resemble strain B or BB (b). Since such effects are solely produced by cytoplasm of the egg, they are described as maternal inheritance (uniparental inheritance).

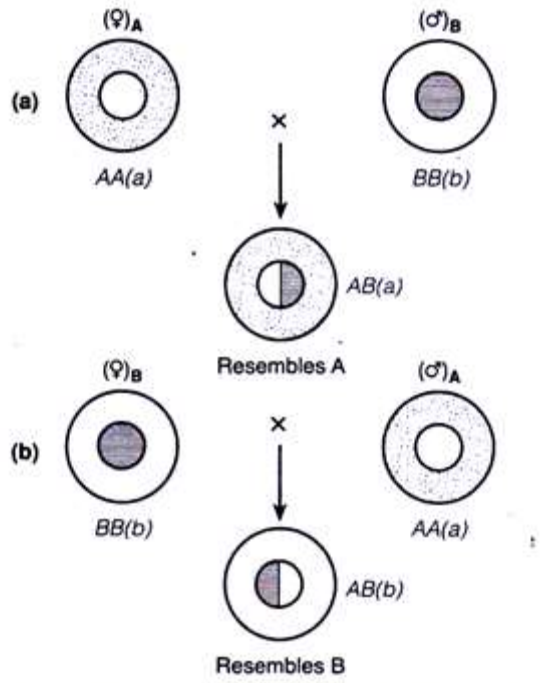


Fig. 10.1: Maternal inheritance showing difference in reciprocal crosses, (a) ♀ A × ♂ B, (b) ♀ B × ♂ A

2. Lack of Segregation:

Irregular Segregation; Somatic Segregation; Lack of Mendelian segregation and characteristic Mendelian ratios that depend on chromosomal transmission in meiosis would suggest extra-chromosomal transmission. Cytoplasmic genes inherited from both the parents sometimes give rise to irregular segregation ratios. They generally show somatic segregation during mitosis, a feature uncommon to nuclear genes.

3. Lack of Chromosomal Location:

The chromosomal genes occupy particular loci and specifically linked to other genes. The failure to find linkage to known nuclear genes may rule out chromosomal inheritance and suggests extra-nuclear inheritance.

4. Association with Organellar DNA:

The cytoplasmic inheritance or extra-chromosomal inheritance is defined as non-Mendelian inheritance, usually involving DNA in replicating cytoplasmic organelles, such as mitochondria and chloroplastids. The presence of DNA in cell- organelles, found outside the nucleus, is a strong evidence to suggest that genetic information does exist in cytoplasm also.

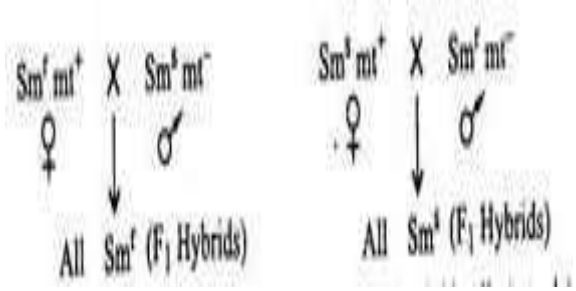
5. Transfer of Nuclear Genome through Backcrosses:

The transfer of nucleus of a variety to the cytoplasm' of another variety through repeated backcrossing, results in lines having nucleus and cytoplasm from two different varieties. A comparison of these lines with original lines having nucleus and cytoplasm of same variety demonstrates cytoplasmic effects on these traits.

Streptomycin Resistance in Chlamydomonas.

Ruthsagar (1965) has reported some cases of extrachromosomal inheritance in green alga *Chlamydomonas reinhardi*. The alga reproduces by asexual as well as sexual means. It does not have different sexes but has positive and negative strains or mating types (mt^+ and mt). The sexual reproduction involves fusion between two morphologically similar but physiologically dissimilar gametes of two different mating types (+ strain or mt^+ and – strain or mt^-) and the gametic fusion results in zygote. The sex is determined by a single chromosomal gene. When meiosis occurs in zygote, four haploid daughter protoplasts are formed which give rise to new plants. Out of 4 new plants resulted from a zygote two are of + strain and the other two are of negative (-) strain. Although both the sexes contribute equally to the zygote, there is maternal transmission of certain cytoplasmic traits. Ruthsagar (1965) isolated two strains of *Chlamydomonas*: one strain was resistant (Sm^r) to 500 jig of streptomycin per ml. of culture solution and the other was sensitive (Sm^s).

When the reciprocal crosses were made between the streptomycin resistant (Sm^r) and streptomycin sensitive (Sm^s) strains, the following results were obtained:



The diploid cells undergo meiosis and give rise to four haploid cells (tetrads) as shown in Fig. 18.3.

From these crosses the following two inferences with respect to Sm resistance can be drawn:

1. The F_1, reciprocal crosses differ from each other.
2. The phenotype of F_1, is governed by mt^+ strain i.e., it is maternal inheritance.

Using the analogy of higher organisms, mt^+ is referred to as female and mt^- as male. The mating type genes mt^+ and mt^- segregate in 1: 1 ratio as expected for the Mendelian inheritance. In the higher organisms formation of zygote involves fusion between an egg and a sperm and the contribution of cytoplasm to the zygote by sperm is negligible. Under such condition it is easy to comprehend the mechanism of maternal inheritance.

But in Chlamydomonas, male (mt^-) and female (mt^+) gametes being identical in size contribute equal amount of cytoplasm to the zygote, even then the cytoplasmic features of only mt^+ strain is expressed in F_1, i.e., it is uniparental inheritance. Now the question arises, what happens to the cytoplasmic determinants of mt^- gametes. This problem was solved by ruthsagar who discovered that the chloroplast DNA of mt^- strain becomes degraded in zygote and the mt^+ gene or a gene closely associated to it specifies a restriction-modification system. Here restriction implies degradation and the modification means protection.

The system encoding the DNA modifying enzyme, modifies its own DNA which cannot be degraded by the restriction system. The mt^+ chloroplast DNA which is not modified or protected is degraded by restriction system of mt^+ gamete. It is suggested that mt^+ linked gene encodes an endonuclease enzyme (enzyme which degrades DNA) which differentiates plastid DNA of its own cell from that of mt^- cell or digested due to modification.

Since plastid DNA of mt^- strain is degraded after sexual union, no expression of mt^- chloroplast DNA is possible and hence uniparental pattern of inheritance is observed. In rare cases (one in a thousand), however, the plastid DNA of mt^- cell escapes degradation by restriction system of mt^+ cell and the zygote contains the plastid DNAs from both the cells. Such a zygote is referred to as cytohet or cytoplasmic heterozygote. Cytohets are important from the view point of studying the recombination of cytoplasmic genes. Ruthsagar has constructed the genetic map of *Chlamydomonas* chloroplast DNA using the genie analysis of cytohets. This suggests that the inheritance of streptomycin resistance is uniparental and the factor for streptomycin resistance resides in the cytoplasm of + strain or mt^+ (Fig 18.3).

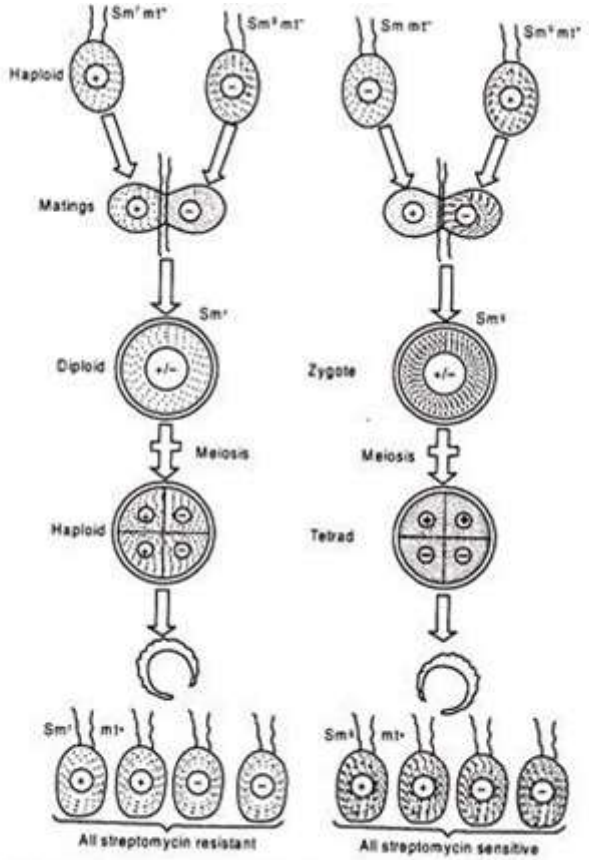


Fig. 18.3 Inheritance of resistance to streptomycin. The plus and minus signs refer to mating types (mt) which is inherited as a single gene difference. The progeny is with rare exceptions always like the plus parent in its reaction to streptomycin, but in these crosses the mating type difference segregates in every tetrad.

Kappa Particles in *Paramoecium*:

One of the most striking and spectacular example of cytoplasmic inheritance due to symbiont bacteria is noted in the most common ciliate protozoa *Paramoecium aurelia*. In 1943, T. M. Sonneborn reported that some strains of P. aurelia contain kappa particles and are known as killer strain.

Kappa particles are the symbiont bacteria called *Caedobacter taeniospiralis*. The diameter of kappa particles are about 0.2µ. They are bounded by a membrane and contain a little bit of cytoplasm with DNA. The strain of *Parameocium* in which the kappa particles are absent are called sensitive strain. The sensitive strains are killed by the killer strain. The destruction of sensitive strain occurs through secretion of a toxic substance called paramecin. This toxic substance is believed to breakdown the food vacuole membrane of the sensitive strain. Paramecin is diffusible in the liquid medium (Fig. 22.8).

When killers are allowed to remain in a medium for a time, they are not killed. It means that paramecin has no effect on killers. Paramecin is associated with a particular kind of kappa that occurs in about 20 percent of a kappa population.

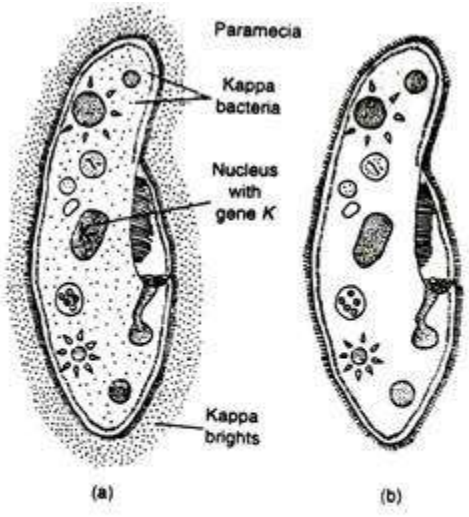


Fig. 22.8: (a) Killer strain Paramoecium with Kappa particles and nucleus with gene K; (b) Sensitive Paramoecium with no Kappa particles and nucleus with gene k.

These kappa bacteria possess a refractile protein containing 'R' body and are called brights because they are infected with a virus that controls the synthesis of a viral protein as well as R protein body in kappa bacterium. The virus may act as the toxin in the killing response and R body facilitates the penetration of the toxin. The non-bright kappa bacteria may also contain virus but the virus may be in provirus state in them.

The killer character of *Paramoecium* has a nuclear as well as cytoplasmic basis. The existence of kappa particles is determined by presence of a nuclear dominant gene K. Kappa particles, like other bacteria, multiply through fission. But their multiplication in the cytoplasm of *Paramoecium* depends on the presence of a dominant nuclear gene K which helps to make an environment necessary for the bacteria to reproduce. When killer strain of *Paramoecium* conjugates with sensitive strain under appropriate condition for brief period and no cytoplasm exchange occurs, two kinds of clones result- one from the original killer cell which contains allele K (Kk) and kappa particles and the other from the original sensitive cell which carries the allele K (Kk) and lacks kappa particles. It indicates that homozygous (either KK or kk) strains become heterozygous following an exchange of K and k genes without cytoplasmic exchange.

Following autogamy (a process of self- fertilisation within one undivided cell resulting in homozygosity), half the progeny (50%) are sensitive *Paramecia*. But all progenies of sensitives following autogamy will be sensitive.

In this conjugation, following autogamy of killers, 50% progeny will receive Kk genotype with cytoplasmic kappa particles other 50% progeny will receive kk genotype with cytoplasmic kappa particles. But it will be sensitive, because kappa cannot reproduce in the cells unless a K allele is present in the nucleus and, as a consequence the kappa are eliminated.

On the other hand, in this conjugation the product of autogamy of sensitive strain obtained after conjugation are all sensitive. All through, 50% progeny of autogamy have KK genotype without cytoplasmic kappa particles because no cytoplasm has been transferred in this conjugation. Remaining 50% progeny of autogamy of sensitive's have kk genotype and no cytoplasmic kappa particles. Under some conditions of conjugation persists much longer; a long connection is established between conjugants (killer and sensitive). In this conjugation, cytoplasm as well as nuclear genes are exchanged (Fig. 22.9). As a consequence both ex-conjugants will receive the genotype Kk and the cytoplasm with kappa particles.

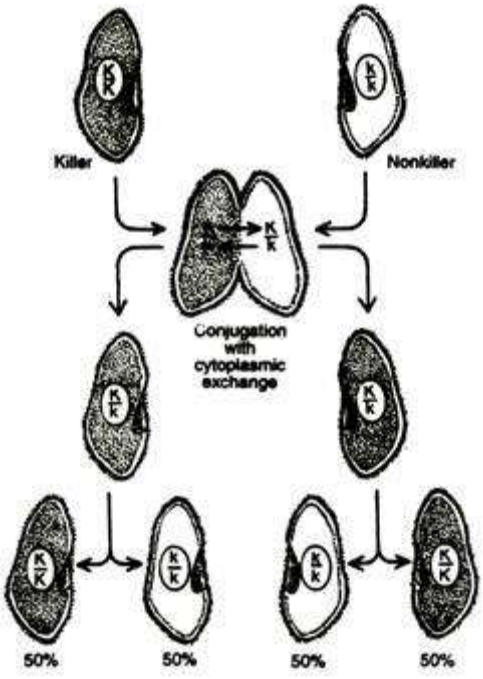


Fig. 22.9: Effect of conjugation for longer period with cytoplasmic exchange followed by autogamy.

Therefore, conjugation for longer period with cytoplasmic exchange will produce all killer strains. Autogamy of both ex-conjugants produces homozygotes KK (killer) and kk (sensitive) cell in the 1 : 1 ratios, respectively, as expected from Mendelian segregation. Therefore, conjugation for shorter period without cytoplasmic exchange does not follow the Mendelian pattern of inheritance. Hence it confirms the cytoplasmic basis of inheritance of killer trait.

Mu Particles in *Paramoecium*:

There is another type of killer trait found in certain strain of *Paramoecium* due to presence of 'mu' particles in the cytoplasm. A *Paramoecium* with a 'mu' particle is called mate killer. On the other hand, a *Paramoecium* having no 'mu' particles is called mate sensitive.

It is so named because when a *Paramoecium* with 'mu' particle conjugates with a partner *Paramoecium* without 'mu' particle then the former kills the latter. The 'mu' particles exist only in those cells whose micronucleus contains at least one dominant of either of the two pairs of unlinked chromosomal genes such as M_1 and M_2. The 'mu' particles are symbionts which are made of DNA, RNA and other substances. The maintenance of the 'mu' symbiont in a *Paramoecium* is dependent upon the genotype of the *Paramoecium*. In fact, the mate-killers of few genotypes maintain their normal number of particles for about seven generations. From the eighth generation, the particles suddenly and completely disappear from the a small fraction of the cell.

Gibson and Beale (1962) suggested that the maintenance of 'mu' particle in *Paramoecium* was due to the presence of another cytoplasmic particle called metagon. It is possibly a long-lived messenger RNA or informosome and may be a product of M_1 and M_2 gene. One metagon may be necessary for the maintenance of hundred 'mu' particles.

Probable Questions:

1. What is cytoplasmic inheritance? How it differs from nuclear inheritance?
2. What are the characteristics of cytoplasmic inheritance?
3. What are the differences between Nuclear Traits and Extra-Nuclear Traits?
4. How streptomycin resistance occur in *Chlamydomonas*?
5. How kappa particle is maternally inherited in *Paramecium*?
6. Describe the role of Mu Particles in *Paramoecium ?*

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7^{th} Edition.

DISCLAIMER: This Self Learning Material (SLM) has been compiled from various authentic books, Journals articles, e-journals and other web sources.

ZOOLOGY

(M. Sc. PROGRAMME)

SEMESTER-IV

ELECTIVE THEORY PAPER:

CELL AND DEVELOPMENTAL BIOLOGY

ZET-404

DIRECTORATE OF OPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

W.B., INDIA

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and coordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE THEORY

CELL AND DEVELOPMENTAL BIOLOGY (ZET 404)

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 404 (Cell and Developmental Biology)	I	Physiology of cell division: Cell Cycle, synchrony in cell division, inhibition of cell division, source of energy.	2	1	1	5-27
	II	Cell signalling: General principles, role of cell surface receptors in cell signalling.		1	1	28-43
	III	Cancer: Characteristics of tumor cells; Oncogenes and their proteins,		1	1	44-57
	IV	Classification and characteristics of chemical carcinogen; role of radiation and DNA repair in carcinogenesis.		1	1	58-72
	V	Cell -cell adhesion: types of cell binding, adhesive proteins, their role in cell-cell interaction		1	1	73-87
	VI	Morphogenesis, differentiation movement of leucocytes into tissues.		1	1	88-95
	VII	Molecular neuron biology; General organization of nerve fibers, Axon Ultra structure, Neurotubules and neurofilaments.		1	1	96-115
	VIII	Neurosecretary cell: Occurrence, staining behavior, neurosecretion in invertebrates		1	1	116-120

Unit I

Physiology of cell division: Cell Cycle, synchrony in cell division, inhibition of cell division, source of energy

Objective: In this unit you will know about physiology of cell division: cell cycle, synchrony in cell division, inhibition of cell division, source of energy.

Introduction

All living organisms of the biological world start life as one cell, i.e., unicellular zygote, the product of the union of gametes - a sperm and an egg. Of course, unicellular organisms live their entire lives as one cell. But in a multicellular organism, the unicellular zygote undergoes countless divisions and produces many cells.

These cells ultimately build the organism to a level of cellular complexity and organisation. The process by which any cell produces its own replica is known as cell division. Thus, simply by cell division a zygote enables an organism to grow. During this period of growth, many cells undergo a course of specialisation that commits them to perform specific functions.

Some cells function in cell division—either they divide to produce gametes for sexual reproduction or they divide to make new cells for growth or to replace old and damaged cells. Thus cell division is at the core of life itself. It helps organisms to grow, reproduce and repair damaged and worn tissue—three fundamental activities of life.

New cells originate only from other living cells. In 1850, Virchow enunciated that every cell originates through division of pre-existing cells. The cell that undergoes division is termed a mother cell, while the cells derived from the division of a mother cell are known as daughter cells. The mother cell transmits copies of its hereditary material in the form of DNA or chromosome to its daughter cells, the next cell generation of cells.

For hereditary information to be transmitted from generation to generation, DNA must be replicated before the cells divide so that each new daughter cell receives a complete copy of hereditary instruction. Since DNA is a part of a eukaryotic cell's chromosome, the chromosomes duplicate as well. After chromosomal duplication, the rest of the division activities proceed in a way that ensures each daughter cell receives the same share of genetic information as well as almost equal proportion of the cell's cytoplasm and organelles. Therefore, in order to divide, a cell must double its mass and increase in shape and size. Cells generally divide when they attain the maximum size.

Phases of Cell Cycle

Most cells divide one or more times during their life time. When they do, they pass through an ordered sequence of events that collectively forms the cell cycle. The duration of the cell cycle varies greatly from one cell to another. The shortest cell cycle occurs in early embryo and can last as little as 8 minutes. The cell cycle of growing eukaryotic cell lasts from 90 minutes to more than 24 hours, its duration varying considerably within a population of cells.

The cell cycle of the eukaryotic cell is divided into two fundamental parts:

i. *Interphase*, and

ii. *Mitosis* (including Cytokinesis)

Interphase is the period of non-apparent division whereas mitosis is the period of division. Actually, for many years cell biologists were concerned with the period of division in which changes visible under the compound microscope could be observed—whereas during interphase no visible changes under compound microscope were seen.

Even chromosomes were not visible in the interphase because the refractive index of the nuclear sap and that of the chromosome present in re-condensed, hydrated and dispraised state become identical. The whole nucleus appears as idle. So interphase was mistakenly considered as resting stage. During interphase of nucleus several changes take place at the molecular level that are not visible microscopically. Interphase is a period of intense biosynthetic activity in which the cell doubles in size and duplicates precisely its chromosome complement. So this phase is also known as metabolic phase and the nucleus is known as the metabolic nucleus.

i) Interphase

The time from the end of one mitosis to the start of the next mitosis is called interphase. It is the phase between the end of last telophase and subsequent prophase. This is the longest period in cell division. Feulgen staining of metabolic nucleus followed by a cytophotometric quantitative assay first suggested that doubling of DNA takes place during interphase.

Auto-radiographic studies with labelled thymine demonstrated that doubling of DNA—i.e., replication or synthesis of DNA— did not take place throughout the entire interphase. It occurs only in a restricted portion of the interphase—the so called S phase, i.e., synthetic period. This period is preceded and followed by two gap periods of interphase (G_1 and G_2) in which there is no DNA synthesis. Thus the interphase can be subdivided into three successive sub-phases G_1,S and G_2 and it normally comprises 90% or more of the total cell cycle (Fig. 1).

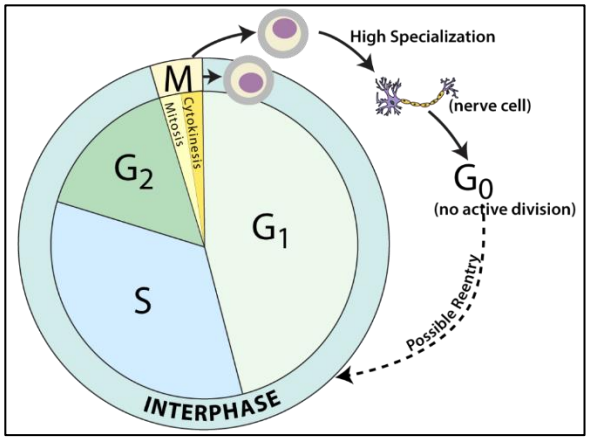

Fig 1: Diagrammatic representation of cell cycle

(a) G_1 Phase

The period between the end of telophase and just before the entry S phase is called G_1 phase. The period of G_1 is usually greater and is subjected to greater variation. Generally speaking, the S and G_1 and mitotic periods are relatively constant in the cells of the same organism. But the G_1 period is the most variable in length.

It may constitute 25-50% of the total interphase duration. In some cells G_1 may be very short or absent. Depending on the physiological condition of the cell it may retain in G_1 phase for days, months or years. Cells that have stopped growing also become arrested at a specific point of G_1 (e.g., liver cell, lymphocytes etc.) and the cells contain the amount of DNA present in G_1 period.

Arrested cells can be induced to divide again. For example, liver cells normally neither grow nor divide but liver damage rapidly induces them to divide. Intensive cellular synthesis takes place during G_1 phase. Mitochondria, chloroplasts, endoplasmic reticulum, lysosomes, Golgi apparatus, vacuoles and vesicles are produced.

In cells preparing for cell division there is a marked synthesis of mRNA, tRNA and proteins during G_1 but there is no DNA synthesis (Fig 2). The enzymes and substrates necessary for DNA synthesis during S phase are also synthesised during this phase. Nucleolus produces rRNA and ribosomes are synthesised. This is necessary for the entry of cells into mitosis as inhibition of their production delays the entry of the cell into mitosis. As a whole, cellular metabolic rate is very high in G_1. As a result, cell growth occurs.

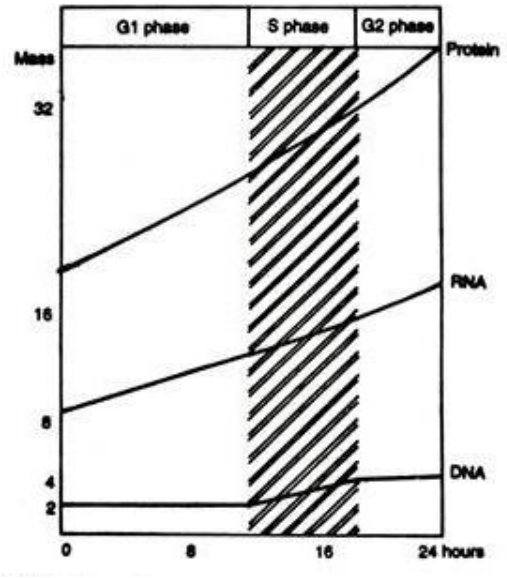

Fig 2: Synthesis of RNA and proteins occurs continuously, but DNA synthesis occurs only in the discrete period of S phase.

Commitment to chromosome or DNA replication in S phase occurs in G_1 phase. If conditions to pass the commitment point are satisfied, after a lag a cell will enter S phase. The conditions mean the nutritional state of the medium, the mass of the cell etc. The commitment point is clearly observed in yeast cell where it is called start. The comparable feature of the animal cell is called the restriction point.

(b) G_0 State

Some cells do not divide at all. These cells are often considered to have indefinitely withdrawn from the cell cycle into another state, resembling G_1 but distinct from it because they are not able to go to S phase, i.e., cells are arrested to non-cycling state.

This non-cycling state is called G_0 state or resting state and the cells are called resting cells. Some cells such as neurons have left the cell cycle irreversibly and can never divide again. But certain types of cell can be stimulated to leave Go and re-enter a cell cycle. For example, liver cells normally neither grow nor divide, but liver damage rapidly induces them to divide.

Indefinite withdrawal from or reactivation into the cell cycle takes place effectively at an early part G_1 phase. The absence of nutrients or growth factors cause cells to enter a resting state. Yeast cells starved of nutrients or mammalian cells deprived of growth factors arrest early in G_1 in the stage G_0.

G_0 cells usually contain fewer ribosomes and less RNA than the corresponding cycling G_1 cells and they synthesise protein less than half the G_1rate. When a G_0 cell is stimulated to grow by growth factor or by providing nutrients, changes in the rate of protein synthesis generally go hand in hand with effect on the chromosome cycle.

The coupling between protein synthesis and the chromosome cycle is not always rigid. With suitable combination of protein synthesis inhibitors and growth factors, it is possible to depress protein synthesis in cultured cells without delaying progress through the cell cycle or conversely stimulate protein synthesis without stimulating cell division.

Comparison of the size of a mammalian neuron and a lymphocyte reveal that both contain the same amount of DNA, but a neuron grows progressively larger during its development while remaining in a Go state. During this time the ratio of cytoplasm to DNA increases enormously. On the other hand, lymphocyte maintains its constant cell size and the definite cytoplasmic ratio by means of cell division.

(c) S Phase

S phase is the intermediate phase between G_1 and G_2 phases. When G_1 phase ends, S phase starts. It is a highly specialised phase of interphase and the word S stands for synthesis. Actually, DNA synthesis takes place in this phase. Before a cell can divide, it must produce a new copy of its chromosomes.

For making a new copy of chromosome it needs both the replication of the long DNA molecule in each chromosome and the assembly of a new set of chromosomal proteins onto the DNA to form chromatin or chromatid.

By its end each chromosome has been copied to two complete chromatids which remain joined together at their centromeres until the M phase that soon follows.

(d) G_2 Phase

The period from the end of S phase until mitosis is called G_2 phase. G_2 phase is usually the shortest part of interphase. In this phase intensive cellular synthesis occurs. Mitochondria and chloroplasts divide. Energy stores increase.

Mitotic spindle begins to form. In the interphase there are two control points such as G_1/S and G_2/M at which the cell takes a decision on whether to proceed or not to the next step. Two control points are also called check points.

This provides an opportunity for the cells to ensure:

a. Whether all conditions are favourable for DNA replication or not?

b. Whether the cytoplasmic mass has increased to a level adequate for division or not.

c. Whether replication has been completed and thus DNA is undamaged.

If the check points did not give any green signal, the cells may halt in G_1/S or G_2/M. Some embryonic cycles bypass some of these controls at some stages of embryogenesis. Thus, the control of the cell cycle can be coupled as required to time, growth rate, mass and the completion of replication.

ii) M-Phase or the Phase of Division:

During this phase the cell divides. During cell division, the nucleus divides first (karyokinesis), which is followed by the division of cytoplasm (cytokinesis).

Division of the Nucleus of a Cell (Karyokinesis): The mitosis is a part of somatic cell division which includes the division of the nucleus (called mitosis or karyokinesis) and the division of the cytoplasm (called cytokinesis).

In mitosis, the metabolic nucleus passes through a complicated system of changes in the form of four different stages, viz., prophase, metaphase, anaphase and telophase. Some important aspects of all these stages are discussed below.

i) Prophase

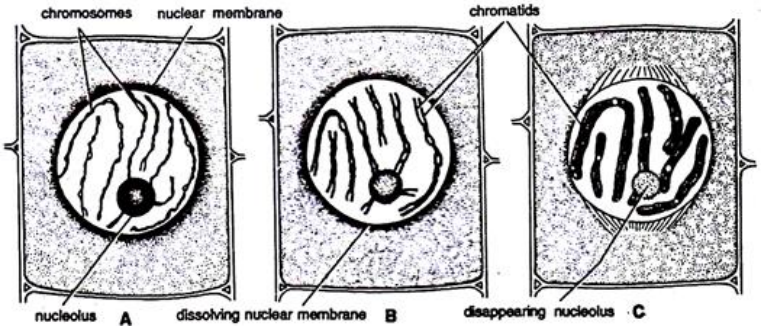

Fig 3: A-B Early prophase stage; C – Late prophase stage

1. It is the first and the longest phase in the mitotic cell division.
2. Chromosomes become visible in the nucleus as short, thick, helically coiled threads (Fig. 3A).
3. Each chromosome splits into two chromatids (Fig. 3B, C) joined at the centromere.
4. Nuclear membrane starts dissolving.
5. Nucleolus also starts dissolving and disappearing.
6. Prophase changes into next stage called metaphase.

4. Chromosomes become shorter and thicker.
5. Chromosomes arrange themselves in the centre or on the equator of spindle.
6. At the end of metaphase, two chromatids of each chromosome also start separating.

ii) Metaphase

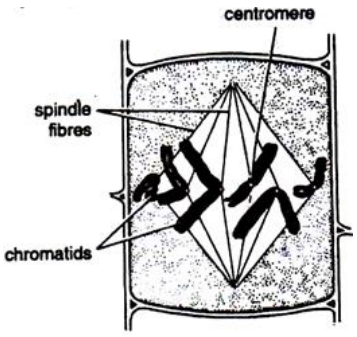

Fig 4: Metaphase stage

1. Nuclear membrane disintegrates and disappears completely (Fig 4).
2. Nucleolus disintegrates and disappears completely.
3. Spindle fibres start appearing and these fibres get attached to chromosomes at centromeres.

4. Chromosomes become shorter and thicker.

5. Chromosomes arrange themselves in the centre or on the equator of spindle.

6. At the end of metaphase, two chromatids of each chromosome also start separating.

7. Metaphase changes into the next stage called anaphase.

iii) Anaphase

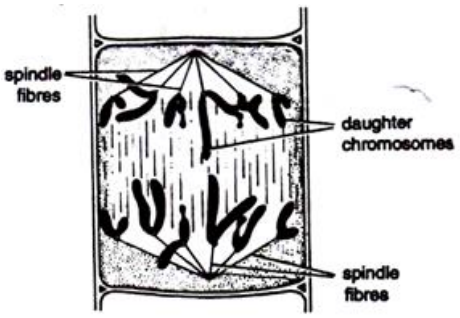

Fig 5: Anaphase stage

1. Chromatids separate from each other at centromere and called daughter chromosomes (Fig 5).

2. Daughter chromosomes move to the opposite poles of the spindle.

3. Daughter chromosomes appear 'V', 'U' or J-shaped during their movement towards poles.

4. Anaphase changes into the next stage called telophase.

iv) Telophase

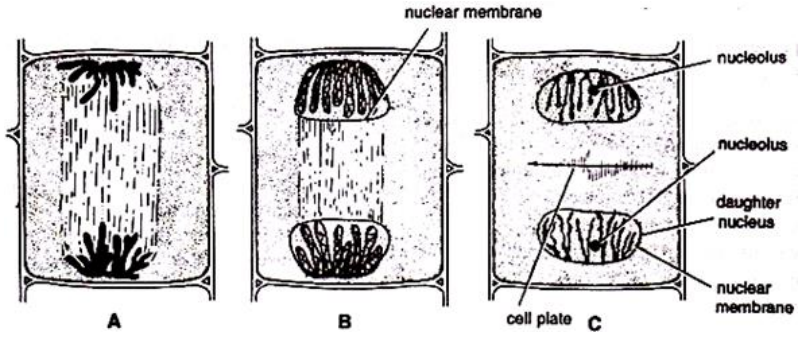

Fig 6: A-C various stages of telophase

1. Daughter chromosomes are now at the end of the spindle, i.e., present on two opposite poles (Fig 6A).

2. Nuclear membrane reforms around each group of daughter chromosomes (Fig 6B).

3. Nucleolus reforms (Fig 6C).

4. Two nuclei are thus organised, one at each pole of the parent cell.

5. Chromosomes begin to lose their compact structure.

6. Spindle fibres disappear gradually.

7. Thus formed two daughter nuclei are exactly similar to the parent nucleus.

Cytokinesis: During the end of telophase a furrow is formed in the cell membrane along the equator. This furrow deepens and considerable movement of cytoplasm takes place. Then all on a sudden the cell is pinched into two along the furrow in the equator and the cytoplasmic turbulence ceases (Fig 7).

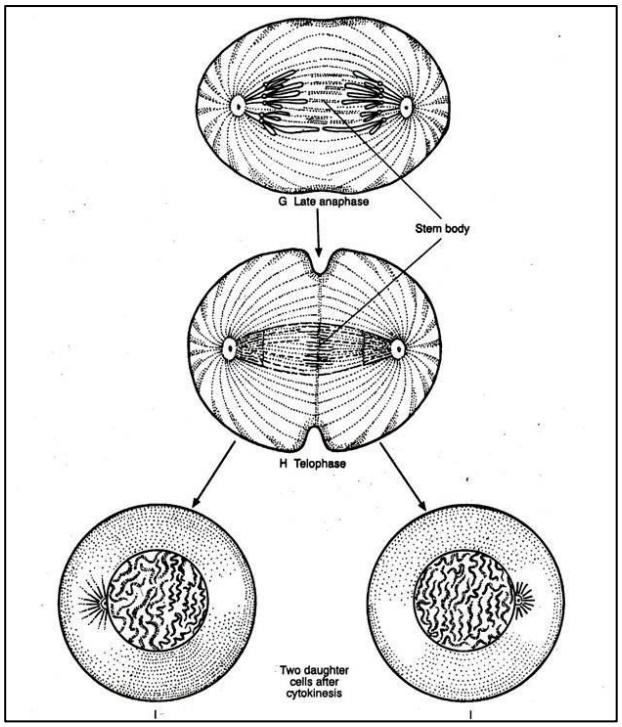

Fig 7: Diagrammatic representation of mitotic phase; G - Late anaphase; H – Telophase; I – Two daughter cells after cytokinesis

Significance of Mitosis

1. Mitosis results in the formation of two daughter cells identical with that of the parental cell.

2. By this process, DNA, the main component of chromosomes, is distributed equally among the two newly formed nuclei.

3. Both the daughter cells formed after mitosis are identical and have the same genetic constitution, qualitatively as well as quantitatively, as the parent cell.

4. The number of chromosomes remains the same from one generation to another generation.

5. Resulted daughter cells have the same characters as were present in the parent cell.

6. The characters of the plants grown by vegetative reproduction may be preserved for a long period.

Regulatory Activities of Cell Cycle

Most important core cell cycle regulators: proteins called cyclins, enzymes called Cdks, and an enzyme complex called the APC/C.

- **Cyclins**

Cyclins are among the most important core cell cycle regulators. Cyclins are a group of related proteins, and there are four basic types found in humans and most other eukaryotes: G_1cyclins, G_1/S cyclins, S cyclins, and M cyclins.

As the names suggest, each cyclin is associated with a particular phase, transition, or set of phases in the cell cycle and helps drive the events of that phase or period. For instance, M cyclin promotes the events of M phase, such as nuclear envelope breakdown and chromosome condensation.The levels of the different cyclins vary considerably across the cell cycle, as shown in Fig 8. A typical cyclin is present at low levels for most of the cycle, but increases strongly at the stage where it's needed. M cyclin, for example, peaks dramatically at the transition from G_2 to M phase. G_1 cyclins are unusual in that they are needed for much of the cell cycle.

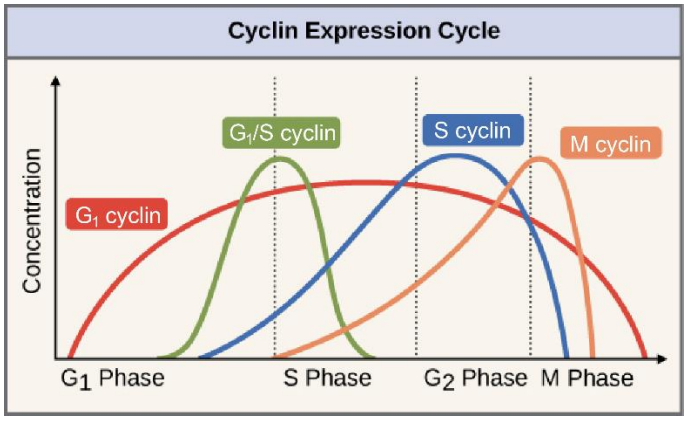

Fig 8: The levels of the different cyclins

- **Cyclin-dependent kinases**

In order to drive the cell cycle forward, a cyclin must activate or inactivate many target proteins inside of the cell. Cyclins drive the events of the cell cycle by partnering with a family of enzymes called the cyclin-dependent kinases (Cdks). A lone Cdk is inactive, but the binding of a cyclin activates it, making it a functional enzyme and allowing it to modify target proteins.

How does this work? Cdks are kinases, enzymes that phosphorylate (attach phosphate groups to) specific target proteins. The attached phosphate group acts like a switch, making the target protein more or less active. When a cyclin attaches to a Cdk, it has two important effects: it activates the Cdk as a kinase, but it also directs the Cdk to a specific set of target proteins, ones appropriate to the cell cycle period controlled by the cyclin. For instance, G_1/S cyclins send Cdks to S phase targets (e.g., promoting DNA replication), while M cyclins send Cdks to M phase targets (e.g., making the nuclear membrane break down) (Fig 9).

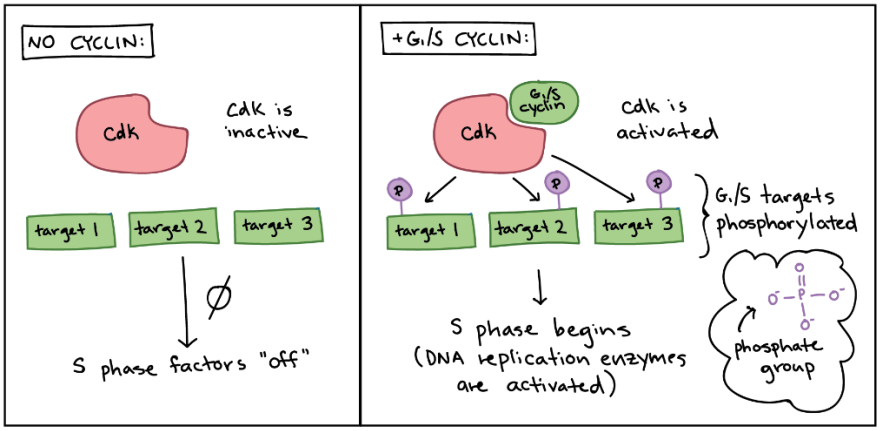

Fig 9: Role of cyclins

In general, Cdk levels remain relatively constant across the cell cycle, but Cdk activity and target proteins change as levels of the various cyclins rise and fall. In addition to needing a cyclin partner, Cdks must also be phosphorylated on a particular site in order to be active and may also be negatively regulated by phosphorylation of other sites.

Cyclins and Cdks are very evolutionarily conserved, meaning that they are found in many different types of species, from yeast to frogs to humans. The details of the system vary a little: for instance, yeast has just one Cdk, while humans and other mammals have multiple Cdks that are used at different stages of the cell cycle. (Yes, this kind of an exception to the "Cdks don't change in levels" rule!) But the basic principles are quite similar, so that Cdks and the different types of cyclins can be found in each species

- **Maturation-promoting factor (MPF)**

A famous example of how cyclins and Cdks work together to control cell cycle transitions is that of maturation-promoting factor (MPF). The name dates back to the 1970s, when researchers found that cells in M phase contained an unknown factor that could force frog egg cells (stuck in G_2 phase) to enter M phase. This mystery molecule, called MPF, was discovered in the 1980s to be a Cdk bound to its M cyclin partner.

MPF provides a good example of how cyclins and Cdks can work together to drive a cell cycle transition. Like a typical cyclin, M cyclin stays at low levels for much of the cell cycle, but builds up as the cell approaches the G_2/M transition. As M cyclin accumulates, it binds to Cdks already present in the cell, forming complexes that are poised to trigger M phase. Once these complexes receive an additional signal (essentially, an all-clear confirming that the cell's DNA is intact), they become active and set the events of M phase in motion.

The MPF complexes add phosphate tags to several different proteins in the nuclear envelope, resulting in its breakdown (a key event of early M phase), and also activate targets that promote chromosome condensation and other M phase events. The role of MPF in nuclear envelope breakdown is shown in simplified form in the Fig 10.

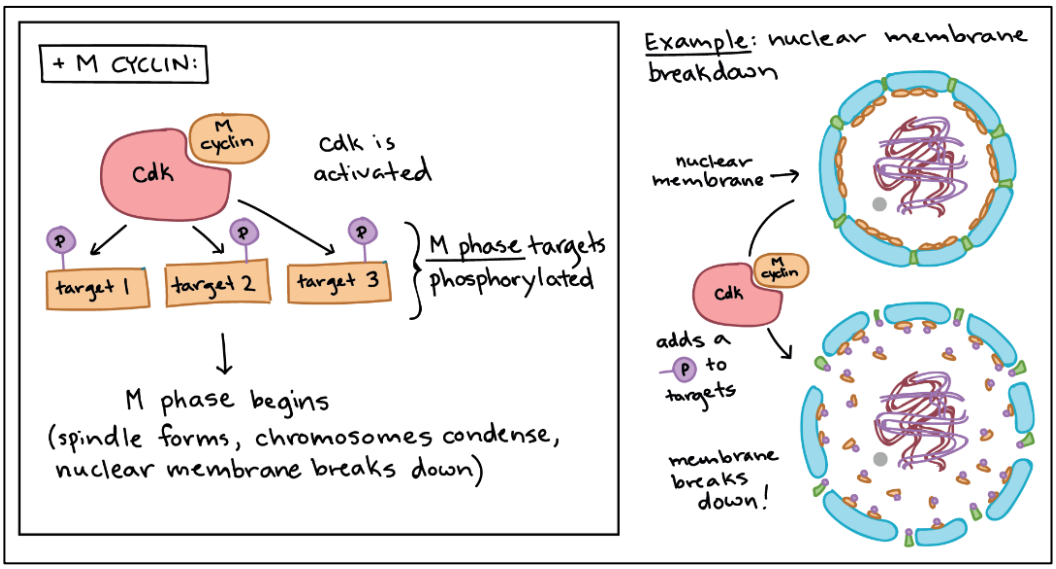

Fig 10: The role of MPF in nuclear envelope breakdown

- **The anaphase-promoting complex/cyclosome (APC/C)**

In addition to driving the events of M phase, MPF also triggers its own destruction by activating the anaphase-promoting complex/cyclosome (APC/C), a protein complex that causes M cyclins to be destroyed starting in anaphase. The destruction of M cyclins pushes the cell out of mitosis, allowing the new daughter cells to enter G_1. The APC/C also causes destruction of the proteins that hold the sister chromatids together, allowing them to separate in anaphase and move to opposite poles of the cell.

Like a Cdk, the APC/C is an enzyme, but it has different type of function than a Cdk (Fig 11). Rather than attaching a phosphate group to its targets, it adds a small protein tag called ubiquitin (Ub). When a target is tagged with ubiquitin, it is sent to the proteasome, which can be thought of as the recycle bin of the cell, and destroyed. For example, the APC/C attaches a ubiquitin tag to M cyclins, causing them to be chopped up by the proteasome and allowing the newly forming daughter cells to enter G_1 phase.

The APC/C also uses ubiquitin tagging to trigger the separation of sister chromatids during mitosis. If the APC/C gets the right signals at metaphase, it sets off a chain of events that destroys cohesin, the protein glue that holds sister chromatids together.

- The APC/C first adds a ubiquitin tag to a protein called securin, sending it for recycling. Securin normally binds to, and inactivates, a protein called separase.
- When securin is sent for recycling, separase becomes active and can do its job. Separase chops up the cohesin that holds sister chromatids together, allowing them to separate.

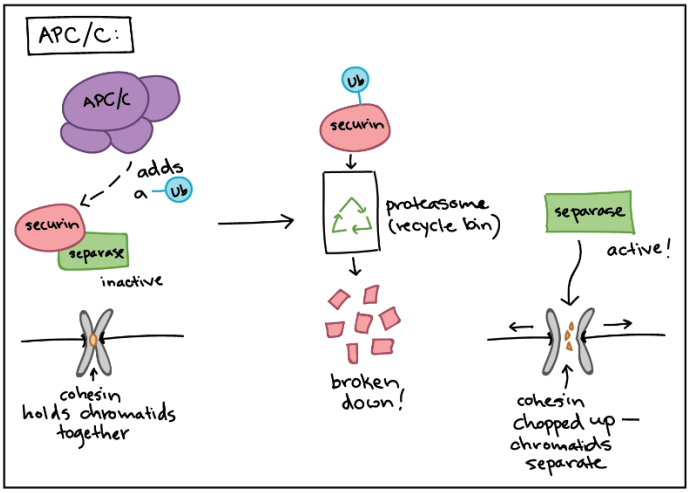

Fig 11: Mode of action of APC/C

Checkpoints and regulators

Cdks, cyclins, and the APC/C are direct regulators of cell cycle transitions, but they aren't always in the driver's seat. Instead, they respond to cues from inside and outside the cell. These cues influence activity of the core regulators to determine whether the cell moves forward in the cell cycle. Positive cues, like growth factors, typically increase activity of Cdks and cyclins, while negative ones, like DNA damage, typically decrease or block activity.

As an example, let's examine how DNA damage halts the cell cycle in G_1. DNA damage can, and will, happen in many cells of the body during a person's lifetime (for example, due to UV rays from

the sun). Cells must be able to deal with this damage, fixing it if possible and preventing cell division if not. Key to the DNA damage response is a protein called p^{53}, a famous tumor suppressor often described as "the guardian of the genome." (Fig 12) p53 works on multiple levels to ensure that cells do not pass on their damaged DNA through cell division. First, it stops the cell cycle at the G_1, checkpoint by triggering production of Cdk inhibitor (CKI) proteins. The CKI proteins bind to Cdk-cyclin complexes and block their activity (see diagram below), buying time for DNA repair. p^{53}'s second job is to activate DNA repair enzymes. If DNA damage is not fixable, p^{53} will play its third and final role: triggering programmed cell death so damaged DNA is not passed on.

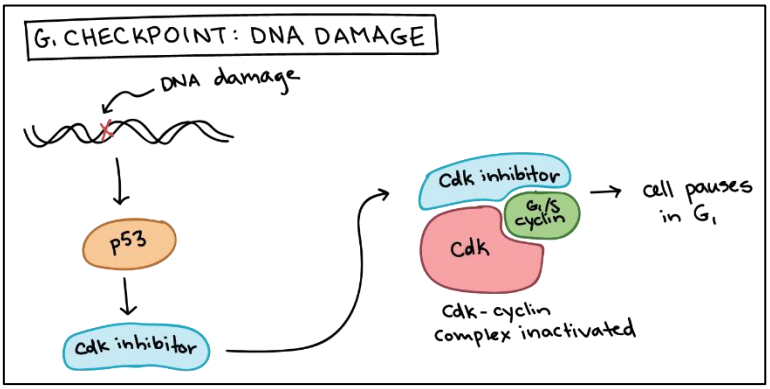

Fig 12: Role of p53

By ensuring that cells don't divide when their DNA is damaged, p^{53} prevents mutations (changes in DNA) from being passed on to daughter cells. When p^{53} is defective or missing, mutations can accumulate quickly, potentially leading to cancer. Indeed, out of all the entire human genome, p^{53} is the single gene most often mutated in cancers. p^{53} and cell cycle regulation are key topics of study for researchers working on new treatments for cancer.

Control of Cell Cycle/ Cell cycle check point

The sequential events of the cell cycle are directed by a distinct cell cycle control system. The signals are transmitted within the cell by signal transduction pathways. Cells have their built in stop signals that halt the cell cycle at checkpoints until overridden by go-ahead signals. A checkpoint in the cell cycle is a critical control point where stop and go-ahead signals regulate the cycle.

Many signals registered at checkpoints come from cellular processes and sometimes the checkpoints register signals from outside the cell. There exists a series of checkpoints which refer to the points of monitoring of cell cycle events such as DNA replication, DNA damage repair, spindle assembly, congression of chromosomes and separation of chromatids/chromosomes to opposite poles, generating signals in case of errors in processes and halting the cell cycle at specified points.

Thus in the cell cycle there are three main types of checkpoints:

(i) DNA damage checkpoint,

(ii) DNA replication checkpoint,

(iii) Spindle checkpoint.

- ***G_1 Checkpoint (DNA Damage Checkpoint)***

It is also called the 'restriction point' and is most important in the cell cycle start phase. If the cell receives a go-ahead signal at the G_1 checkpoint, it usually completes the cell cycle and divides. If it

does not receive the go-ahead signal, the cell exits from the cell cycle and switches to a non- dividing state, the G_0 phase.

This checkpoint monitors damaged DNA which detects DNA damage and does not allow entry of cell into S phase until the damage is repaired. This checkpoint blocks progression into S phase by inhibition of S-Cdk complex. Damaged DNA stimulates transcription of many genes which encode the proteins that bind to S-Cdk, inhibits their activity and thus blocks the entry into mitosis.

In mammals, a protein of p^{53} gene causes delay in entry of cells with damaged DNA into S phage and mutation in p^{53} gene, therefore, causes cancer due to increase in frequency of cancer promoting genetic alterations. The protein p^{53} blocks the activity of Cdks and has been dubbed as 'Watchman' because DNA damage is sensed by it (Fig 13a).

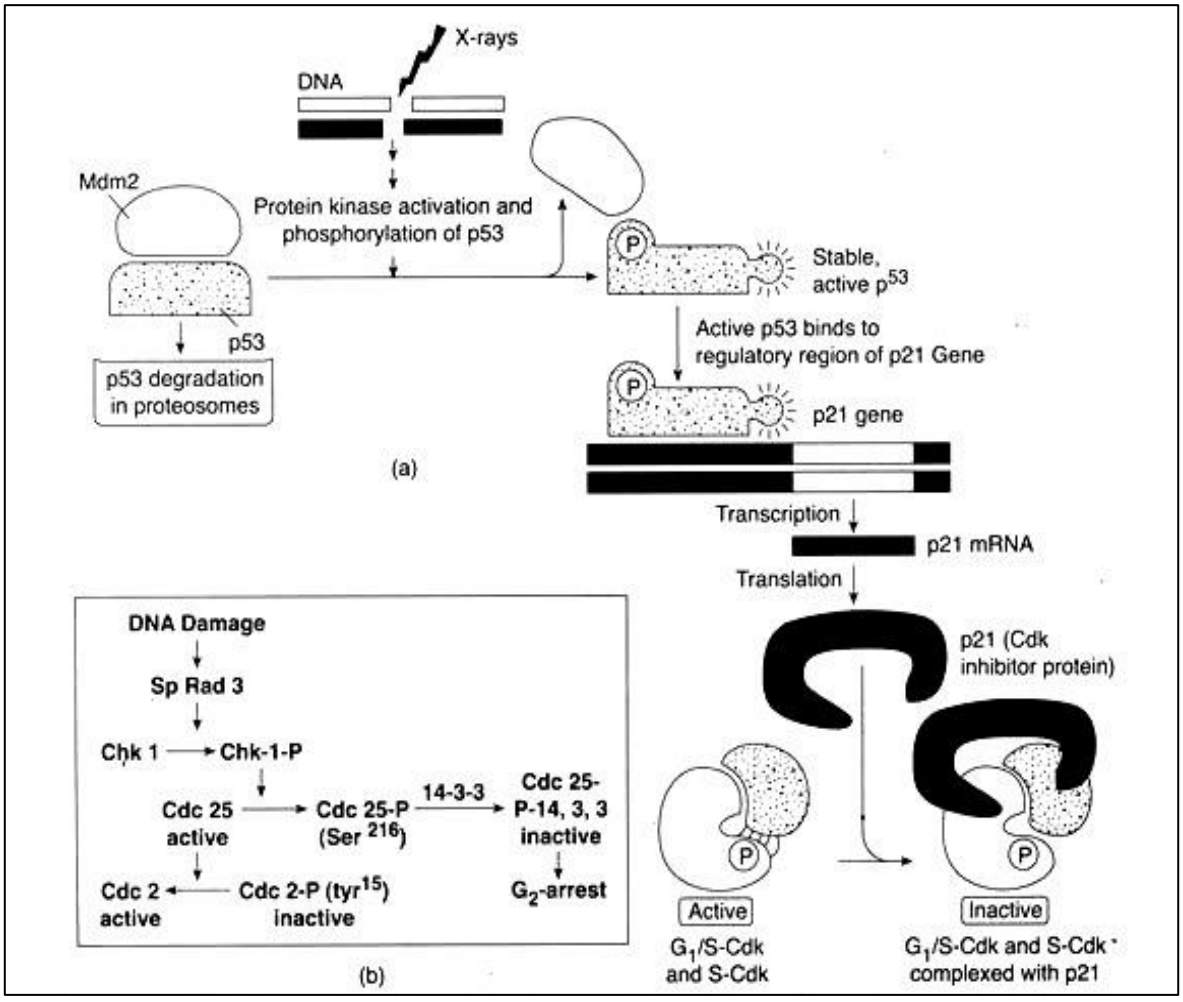

Fig 13: Models showing the operation of (a) DNA damage checkpoint in G_1 (b) DNA damage check point in G_2

- ***G_2 Checkpoint (DNA Replication/DNA Damage Checkpoint)***

It is the controlling point involved in driving the cell to the M phase. This is triggered by MPF (Maturation Promoting Factor or M-phase Promoting Factor) which is a cyclin-Cdk complex (Cdk – cyclin dependent kinase). It promotes mitosis by phosphorylating a variety of other protein kinases.

This checkpoint monitors un-replicated and damaged DNA which delays mitosis until DNA replication is complete and DNA damage is repaired. Mitotic entry of G_1 cells is delayed in yeast by the rad 9 gene.

The damaged DNA sends a signal to a series of protein kinases that blocks the dephosphorylation and activation of M-Cdk, blocking entry into mitosis (Fig 13b). Normal cells treated with hydroxyurea, inhibitor of DNA synthesis, activates this checkpoint mechanism that arrests the cells in S phase, thus delaying mitosis.

- ***M-phase Checkpoint (Spindle Attachment Checkpoint):***

It ensures that all the chromosomes are properly attached to the spindle at the metaphase plate before anaphase. A signal to delay anaphase originates at kinetochore which inhibits attachment of spindle microtubule, this keeps the anaphase promoting complex (APC) in an inactive state.

After attachment of all kinetochores, the APC is activated, triggering breakdown of cyclin and inactivation of proteins holding sister chromatids together. Mutants in MAD and BUB genes of mammals and yeast inactivate this spindle checkpoint (Fig 14); the effect of colchicine which inhibits spindle assembly demonstrate the presence of this checkpoint.

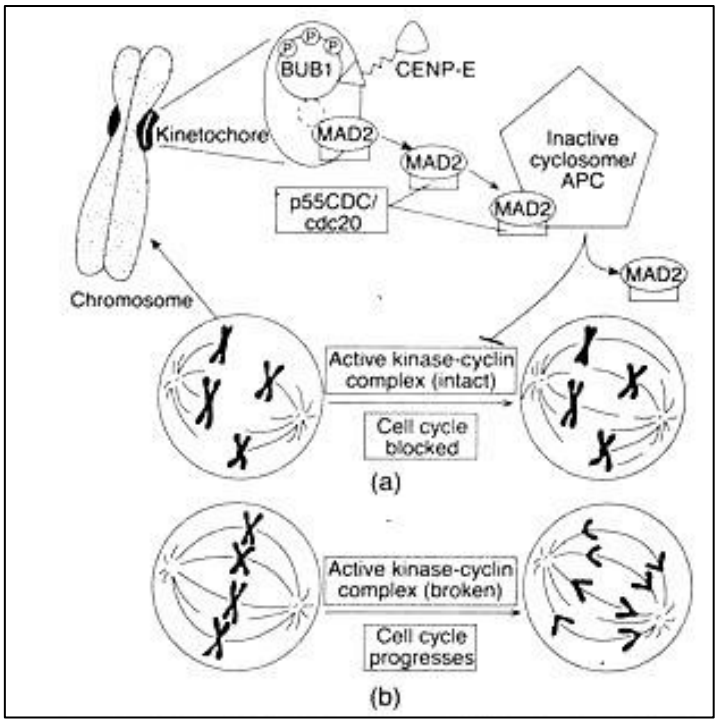

Fig 14: A model showing the operation of spindle assembly checkpoint (a) Chromosomes not aligned on equatorial plate, cell cycle blocked; (b) Chromosomes aligned on equatorial plate leading to separation of chromatids.

Synchrony in cell division

Cell synchronization is a process by which cells in a culture at different stages of the cell cycle are brought to the same phase. Cell synchrony is a vital process in the study of cells progressing through the cell cycle as it allows population-wide data to be collected rather than relying solely on single-cell experiments. The types of synchronization are broadly categorized into two groups;

i) physical fractionization, and
ii) chemical blockade

For many of the studies of cell division it has been found desirable to have all of a population of cells in a culture divide at one time. Such synchronised cell division makes possible more effective analysis of the various components of the process.

Eggs of marine animals have been favourite objects for such studies since practically all the eggs divide at the same time in a suspension of healthy and normal cells. This synchrony in dividing marine eggs implies that some event has occurred to achieve the synchrony, e.g., the almost simultaneous entry of sperm in all eggs starts a train of events that takes about the same amount of time in each of the cells.

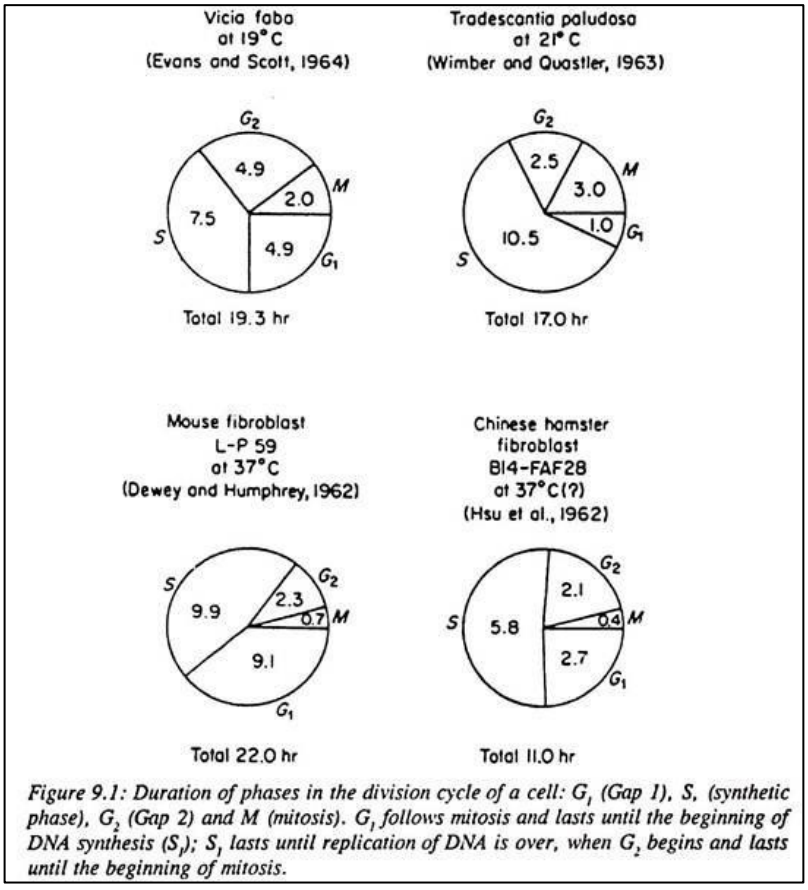

Figure 9.1: Duration of phases in the division cycle of a cell: G_1 (Gap 1), S, (synthetic phase), G_2 (Gap 2) and M (mitosis). G_1 follows mitosis and lasts until the beginning of DNA synthesis (S_1); S_1 lasts until replication of DNA is over, when G_2 begins and lasts until the beginning of mitosis.

Cleavage in the embryo continues in synchrony for several generations unless cells are separated from one another. When the eggs have been affected by unfavourable conditions, e.g., temperature, radiations, pH, division may be delayed, and when it starts, some eggs are found to cleave before others.

The susceptibility of the cells to the unfavourable conditions varies about a mean for the population and a characteristic distribution curve is obtained for onset of division in the population. In a culture of bacteria, protozoa or tissue culture cells the number of cells in division at any one time is limited, being from 5 to 10 per cent. The ratio of the number of cells in division to the total number of cells is called the mitotic index.

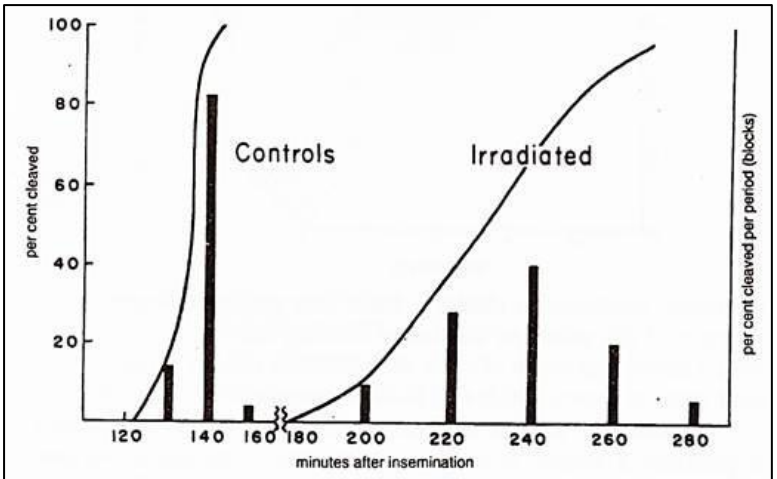

Figure 9.2: High degree of synchronyu in division of normal sea urchin eggs (inseminated simultaneously) as shown in the curve for "controls".

The mitotic index appears to be a function of the generation time for a given species under the conditions provided and the actual length of time a cell of this species remains in mitosis. The mitotic index may be increased by selecting a single cell from such an asynchronous culture and using it as the progenitor of a culture.

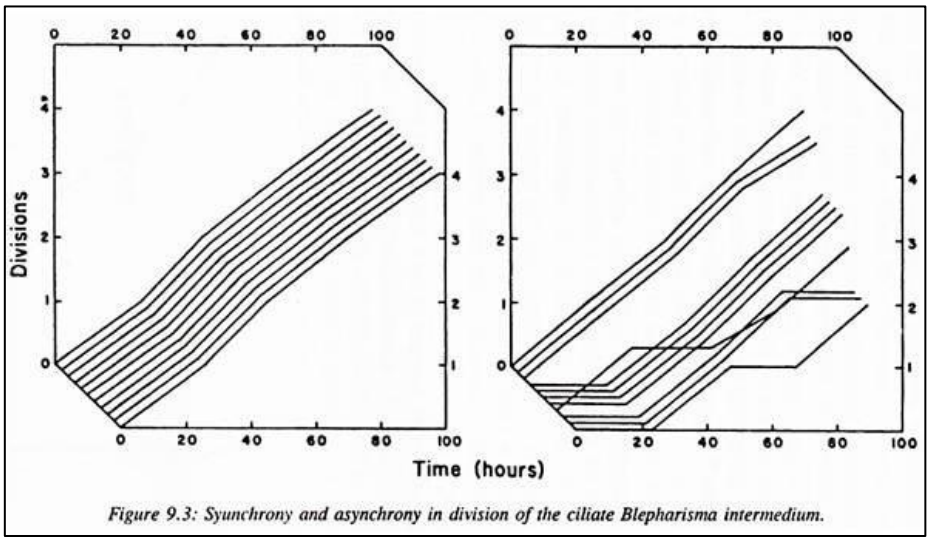

Figure 9.3: Syunchrony and asynchrony in division of the ciliate Blepharisma intermedium.

For a few generations thereafter, synchrony is obtained, but it also gradually disappears. A variation of this technique consists of filtering bacteria of a given size from a mixed population of cells, thus obtaining cells in essentially the same stage of growth which will divide at approximately the same time.

The failure to achieve synchrony of division in a culture of cells is probably a result, in part, of differential exposure to various environmental conditions. In a syncytium (multinucleate cell) in which mixing of all materials occurs and the conditions of the environment are similar, the division of nuclei is usually perfectly synchronised. Also, in the syncytial insect egg nuclear division continues synchronously for as many as eleven generations.

The same is true for the nuclei in the syncytial endosperm of plant embryos. It is thought likely that nuclei in division may secrete division- stimulating materials. To explain the low mitotic index in most cell cultures it has been suggested by some that the division-stimulating material from the few cells in division does not affect many other cells, either because they are distant or because the membranes of such cells are relatively impervious to the hypothetical division-controlling substances.

This concept of control of synchrony by secretion of materials from nuclei has some experimental backing. For example, grafts can be made between two multinucleate amoebas (*Chaos chaos*), each with nuclei synchronously dividing but out of phase with one another. After one cycle the division of all nuclei from both amoebas now present in one cell is synchronous, the larger piece imposing its time upon the smaller.

There are many examples of synchronised cell division which can be found in cells of higher plants and animals. Cells in synchronous division are found, for example, in spermatogenic cells lining the lumen of a germinative tubule, which are connected to one another by cytoplasmic bridges. Such bridges insure entry of the division-stimulating material from one cell to another. It is important at this time to emphasize that a suspension of cells, whether they are cells in tissue culture, protozoans, yeast or bacteria, are fundamentally different from a suspension of marine eggs. Marine eggs have undergone a period of growth in the ovary and they have self- contained supplies which will serve for many cell divisions before intake of nutrients need take place. Cells other than eggs, however, must incorporate nutrients and grow before they can divide. Perhaps not all cells are able to incorporate the same nutrients at the same rate.

Studies on *Tetrahymena* (ciliate protozoan) show that the mass of the cell increases linearly as measured by oxygen consumption, by increase in volume and by ^{14}C-methionine incorporation, during the entire synthetic period, (S), from the onset of furrowing in one division until about 10 to 20 minutes before the next division. The division cycle has a generation time of 2.25 to 2.5 hours at 28 to 29°C.

Furthermore, ^{3}H-histidine incorporation (protein synthesis) and ^{3}H- uridine incorporation (RNA synthesis) continue during division with little change. During the 10 to 20 minute predivision period, which precedes furrowing, there is no increase in mass while the cell gets ready to divide. Such a period has also been found in other cells; e.g., in *Amoeba* it occupies about one-sixth of the generation time.

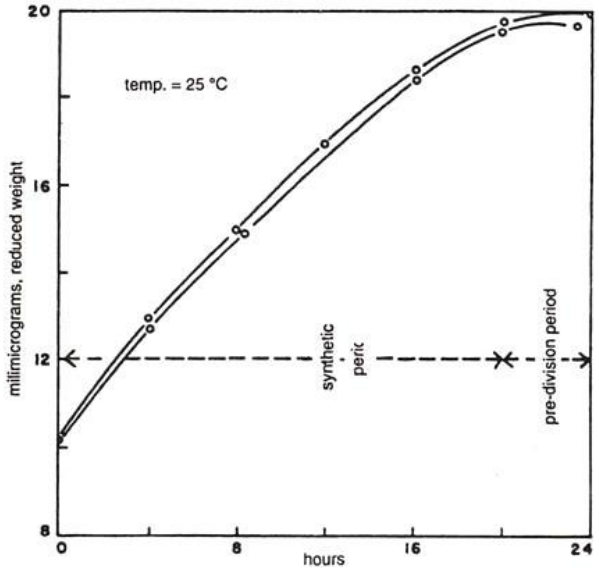

Figure 9.4: Growth in mass of Amoeba proteus. *The pair of lines represents the growth of a pair of sister cells from the time of division of their mother cell to the time they themselves divide.*

Studies on *Paramecium aurelia* show that, the mass, as measured by increase in dry weight, changes little for a period after division, and then it increases almost exponentially. From the data recorded this would appear to be exceptional compared to most cells studied.

On the other hand, Lovlie in a careful study on single *Tetrahymena* (using the diver technique) has shown that the type of curve obtained for increase in mass with time is related to the conditions of growth. He found three types of curves for Tetrahymena, exponential, linear and linear with a plateau, depending on whether the growth was balanced (doubling during the interdivision period) or unbalanced.

Since it is not possible to achieve synchrony of cell division in cell suspensions other than marine eggs, attempts have been made to shock cells into synchrony.

Two methods have been used:

i. Chemical shock and

ii. Physical shock.

Chemical shock consists of withholding or limiting the supply of some nutrient necessary for division and then supplying it to the culture at one time in a large quantity, inducing in this manner a high level of simultaneous metabolic activity. The physical shock is one that is unfavourable for the act of cell division yet favourable to other metabolic activities preceding division, thus allowing the cells in the pre-division stages of the division cycle to, catch up with those in the later stages of the division cycle.

Only a few experiments using shocks for synchronisation of cell divisions will be considered here. Cell division and DNA synthesis in Escherichia coli T-15, a thymine-requiring mutant (thymineless), are blocked immediately upon transfer of the organism to a thymine-free medium. However, RNA and protein synthesis continue, apparently at the original rate. When thymine is added 30 minutes later, DNA synthesis is resumed and nearly all the cells are found to divide simultaneously after a lag of 35 to 40 minutes. Similarly, in Lactobacillus acidophilus, synchrony can be induced by the addition of thymidine to a thymidine-starved culture.

Yeast cells starved in succinate buffer until some of the reserves are gone will divide synchronously after return to complete nutrient medium, including carbohydrate and nitrogen sources. Similar results were obtained with a variety of cells needing some particular metabolite. For example, synchronous division in the cells of the epidermis of the insect *Rhodnius* follows its periodic ingestion of blood. In *Chlorella* and various algae, all photosynthetic, the daily periodic lighting regimen makes possible synchronisation of cell division, presumably by periodic accumulation of food reserves during the period of illumination. Lighting, too, may be considered a physical shock, much like temperature; in fact, all the arguments used for temperature apply to light in light- sensitive cells. Environmental changes produce oscillations in growth of many types of cells.

Use of temperature as a physical shock to obtain synchronised division stems from the notion that the processes that occur during the division cycle are differentially sensitive to temperature. If some reaction in the pre-division period is more sensitive to heat than are the reactions in the synthetic period, then a high temperature might prevent division without stopping syntheses.

Thus, cells lagging behind in preparations for division might be given a chance to catch up with the others. As expected, a single temperature shock synchronises only a small fraction of the cell population because it allows only a small proportion of the cells to accumulate in pre-division stages.

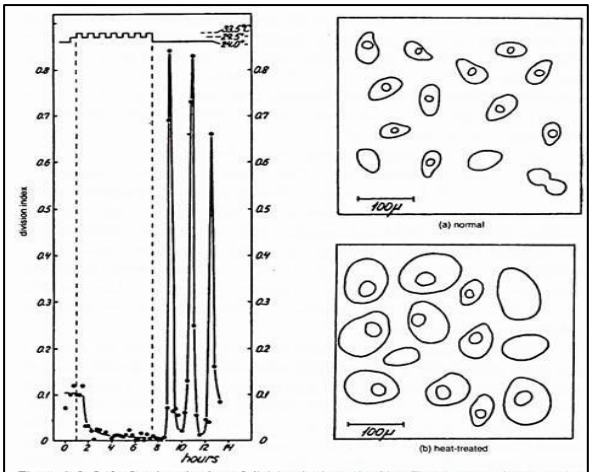

Figure 9.5: Left, Synchronisation of division by heat-shocking Tetrahymena. Seven shocks at 33.5°C. are given in succession, alternating with 29.5° C. At the conclusion of the heat shocks, the temperature is dropped to 24.0°C. The three post heat treatment divisions are synchronised to the extent indicated by the division index. Right (a), Normal Tetrahymena drawn with camera lucida and (b) Tetrahymena after heat treatment. The circles inside the cell are the macronuclei.

On the other hand, a series of temperature shocks which block division-each alternating with an exposure to a near optimal temperature for a period insufficient to allow the cells in the pre-division stages to divide-synchronises most of the cell population, because of the gradual accumulation of a large proportion of the cells in pre-division stages before cessation of heat shocks.

Zeuthen and Scherbaum found that alternate exposure of suspensions of the ciliate *Tetrahymena*, for half-hour periods at 28 to 29°C. (optimal) and 34°C. (inhibitory) for seven cycles resulted in 85 per cent synchronisation of the cells in division when the cultures were subsequently kept at 28°C. Synchrony persisted for several cell generations and then division became random. Thermal shocks have also been effective for synchronising division of many other kinds of cells, including bacteria.

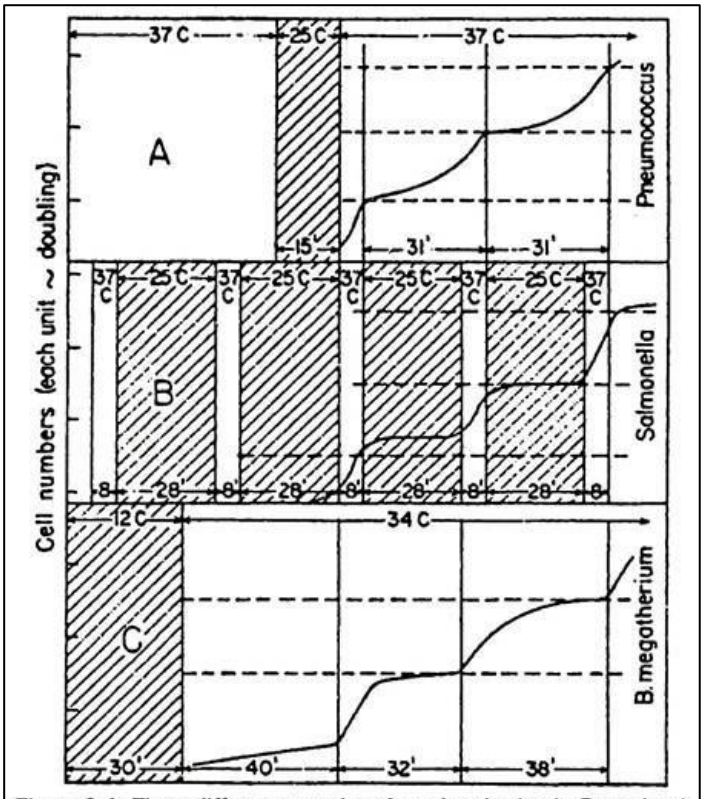

Figure 9.6: Three different examples of synchronisation in Bacteria. A, Pneumococcus. B, Salmonella typhimurium . C, Bacillus megatherium.

Cold shocks have also been used to induce synchronisation of cell division in a number of protozoans and bacteria. Nutritional deficiency, coupled with temperature shocks, has also been very effective. X-rays have also been shown to induce synchrony in division, again presumably by holding back the cells in the radiation-sensitive pre-division stages and causing accumulation of the cells which will ultimately divide at nearly the same time.

Much effort has gone into production of synchronised cultures of cells because in such cultures division and growth are essentially uncoupled. Therefore, growth can be studied in the cells prior to division and the process of division and its accompaniments can then be studied in the cells accumulated in the pre-division stages.

The cells shocked into synchrony have accumulated sufficient reserves for a series of divisions, which follow one another more rapidly than the usual generation time for the species until the accumulated reserves have been partitioned among the descendants.

This, of course, is also true of a suspension of marine eggs. Heat-shocked cells thus grow until they are considerably larger than controls. In a heat-shocked culture of Tetrahymena, the generation time for the first few synchronised divisions is about 60 per cent of that required for a culture that was not subjected to heat shock.

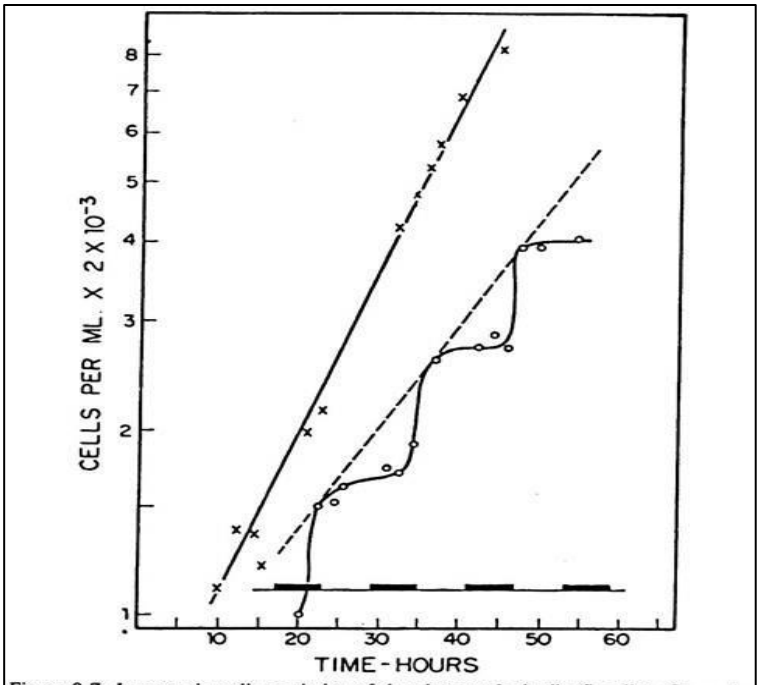

Figure 9.7: Increase in cell population of the photosynthetic dinoflagellate Gonyaulax polyhedra in continuous light (X) and in alternating light and dark periods of 12 hours (open circles). Light intensity, 900 foot-candles; temperature, 21.5° C.

Division of heat-shocked *Tetrahymena* continues even in the presence of inhibitors of synthesis of DNA and steroids. However, certain long chain unsaturated fatty acids must be synthesized, as must certain specific proteins. The specific protein synthesis continues even if the cells are starved.

The specific protein is presumably "division protein" thought to be required for structuring the cell for division. It has been extracted and characterised. Interestingly, DNA synthesis, as measured by ^{3}H-thymidine incorporation is not synchronised with cell division in heat- shocked cultures. It would be of interest to determine the DNA polymerase activity at various times during the cell division cycle.

Synchronised cultures of various cells in suspensions are now being widely used in biochemical and cytochemical research. It is important to note, however, that changes in size and composition of the

cells after synchronisation must be taken into consideration. For example, resistance of such cells to ultraviolet radiations is markedly altered.

Synchronisation of cell division in the photosynthetic dinoflagellate Gonyaulax, in the normal diurnal rhythm of day and night, occurs in 85 per cent of all cells destined to divide during a 5 hour period spanning the end of darkness and the beginning of the light period.

Although it might appear that cell division here, as in Chlorella, is related to periodic changes in nutritional conditions, keeping Gonyaulax in continuous dim light only sufficient to maintain nutritional balance in the cells does not stop synchronous division for at least 14 days.

The synchronous division here is also relatively independent of small variations in light intensity and temperature. In high light intensity, however, synchrony is lost in 4 to 6 days. This is an interesting case worthy of detailed study. At present it is interpreted as an example of inherent biological rhythm.

Bruce lists the generation time of cells from a large number of species of plants and animals. In many cases the division of cells appears to be circadian, almost every 24 hours, but it has not yet been determined whether the day-night cycle will synchronise these to a 24 hour rhythm.

Source of Energy for Cell Division

Cell division, is a fundamental activity of the living cell. During cell division the cell does work at the expense of energy derived from nutrients, as witness the case just cited of synchronised cell division in yeast obtained whenever the cells are supplied with adequate food. It has also been shown that addition of glucose to isolated epidermal tissue culture results in synchronous division of many cells. Presumably, cell division had previously been blocked by lack of nutrient since such cells store little glycogen. Even if glucose is supplied, division fails if oxygen is not available.

Glucose may be replaced by lactate, glutamate, fumarate or citrate. All the experiments suggest that operation of the Krebs cycle supplies the energy for division of the cells. As expected, mitosis is inhibited by Krebs' cycle poisons such as malonate, cyanide and carbon monoxide, and by phlorhizin, a phosphorylation inhibitor.

The latter finding suggests that high energy phosphate bonds are an energy source or are involved in building this source. Marine eggs and protozoans require oxygen for division, but frog eggs and many embryonic tissue cells do not, presumably supplying their energy for cell division by glycolysis. Cells which require oxygen for division have a lower rate of glycolysis compared to their rate of respiration.

Cells which divide in absence of oxygen have rates of glycolysis much higher than that of respiration, sometimes several fold greater. As might be expected, in cells which can supply their energy needs for division by glycolysis cell division is very sensitive to glycolytic inhibitors such as iodoacetic acid. In order to have an effect on cell division the energy sources must be supplied early in the cell division cycle during what Bullough calls the antephase.

Once a critical concentration of the energy-rich substances has been built up and the prophase begins, he found that nothing short of killing the cell will stop it from dividing. This has been noticed not only for cells deprived of oxygen or metabolically poisoned, but also for those damaged by radiations or subjected to other injuries; such cells divide and soon thereafter cytolyse. Initiation of mitosis appears to begin a series of concatenated and irreversible reactions which stop only when the cells have divided.

By employing carbon monoxide, working under green light, to block cytochrome oxidase in sea urchin eggs and releasing the block by shifting from green light (which was not thought to be

absorbed by the enzyme-carbon monoxide complex) to white light, Swann obtained the following results. If the inhibitor is applied before sea urchin eggs enter mitosis, the first cleavage is delayed by a time about equal to the time of application of carbon monoxide.

If the inhibitor is applied after the cells have entered mitosis, they complete mitosis and cleave with little or no delay, but the second cleavage is delayed for a period which is roughly equal to the period of inhibition. It was suggested that these results can best be explained by a hypothetical energy reservoir which is continually being filled and in which energy is stored for a cell division during the preceding mitosis and cleavage.

Once the energy has siphoned out it carries the cell through mitosis and cleavage; if at this time the cell is poisoned for a period by monoxide and energy is no longer being accumulated in the reservoir, cleavage will nevertheless continue to completion. However, the next cell division is delayed for an interval equal to the period of application of the poison. Lack of oxygen and various other poisons that affect the aerobic enzymes would also affect cell division in a similar manner.

Poisons which do not affect energy-liberating systems act in a different manner. For example, ether (1 per cent) is almost without effect if applied to a suspension of sea urchin eggs before the mitotic spindle has formed, but if applied after that time, it blocks development of the spindle fibers, maintaining the proteins in a solvated state.

If the treated eggs are then washed free of the ether, development proceeds at once, the only delay being the period during which the eggs were subjected to the ether since in this case the energy reservoir, if it exists, is unaffected by ether.

The need for a postulated energy reservoir is difficult to accept on the basis of data collected both on animal and plant cells. Epel found that cleavage rate in the eggs of the purple sea urchin was decreased when the concentration of carbon monoxide was at a level which inhibited respiration. At this time the ATP level was also decreased. When the ATP level had dropped to 50 per cent of normal, mitosis was completely inhibited.

By varying the carbon monoxide concentration, and thus obtaining varying degrees of inhibition of ATP production, Epel demonstrated that the mitotic rate paralleled the ATP level in cells. He also found that division could be blocked at any stage of mitosis if the inhibitor was applied at the appropriate time.

When phosphorylation is uncoupled by dinitrophenol (DNP), ATP fails to accumulate and cell division is also blocked. Such a concentration of DNP, which uncouples phosphorylation, causes an increased rate of respiration, but the respiration is now useless and idling.

This point again at ATP as the likely source of energy for cell division. The discrepancy between the results of Epel and those of Swann perhaps rests upon a difference in experimental technique. Swann did his “dark” experiments in green light, which is theoretically not effective in reversal of carbon monoxide poisoning; he used white light for full reversal of carbon monoxide poisoning.

Epel, on the other hand, put his carbon monoxide experiments in full darkness because he found that in green light the rate of respiration and ATP production were reduced to only 75 per cent of the level obtained by the same concentration of carbon monoxide in full darkness.

Heat shock, does not stop growth although it prevents division, and, consequently, the cells may reach a size four times that of the controls. Returning the cells to the optimal temperature after one or more heat shocks permits cleavage in less than the generation time.

Presumably the mitogenic and growth channels are separate at their definitive ends, though both are using the same sources of energy and materials. When the mitogenic channel is blocked by physical or chemical means, the energy and material which might normally go through this channel pass instead into synthetic reactions leading to extra cell growth. Consequently, after removal of the block, sometime must elapse before the specific molecules necessary for cell division are synthesized to the necessary level.

In Tetrahymena if heat shocks as used by Zeuthen continue well beyond seven cycles, the cells may ultimately divide, even during a heat shock. This is taken to mean that accumulation of products of growth in a cell, and especially of the material needed for cell division has reached a point at which division is initiated. Division can then no longer be prevented by thermal shocks that previously blocked it.

Perhaps an even more clear-cut separation of growth processes from cell division is shown in experiments with Tetrahymena cells which are heat shocked in nutrient medium and then transferred to balanced salt medium with no nutrients. Such cells subsequently divide at least twice in absence of nutrients. Cleavage of such cells under these conditions can be "set back" or delayed by various metabolic poisons or physical shocks.

Experiments with dinitrophenol also distinguish two phases of metabolism in Tetrahymena. One, constituting about 30 per cent of total metabolism, relies on endogenous reserves and can support cell division; the other phase (about 70 per cent of total metabolism) depends upon exogenous supplies and is coupled with growth.

It has become increasingly evident that specific protein (division protein) probably plays an important role in cell division in *Tetrahymena*. It is thought that a critical concentration of this particular protein is needed for cell division. It has also been shown that ATP, GTP and RNA accumulate in the period just preceding cytoplasmic division and that the changes are reversed at mitosis.

However, such an accumulation of free energy sources is interpretable on the basis of protein synthesis, because protein synthesis decreases for a period beginning just about two thirds of the time to the next cell division. At this time the protein formed on the ribosomes is less readily released than it was previously.

The call for ATP is then less than its concentration in the cell, along with that of GTP, and RNA therefore increases. This change in concentration thus appears to be incidental to the slowing or cessation of protein synthesis rather than due to accumulation of an energy reservoir. The high energy phosphates then play a part in reversing the binding of the protein to the ribosomes.

Probable questions:

1. Describe the interphase stage of cell cycle in details.
2. Discuss in details about the cell cycle check points.
3. Describe the stages of mitosis cell division with suitable diagram.
4. Discuss the role of cyclins in cell cycle regulation.
5. How DNA damage halts the cell cycle in G_1?
6. Discuss the role of p^{53} in cell cycle regulation.
7. Discuss the synchrony of cell division.
8. Discuss about the energy source in cell cycle event.

Suggested readings:

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Unit II

Cell signalling: General principles, role of cell surface receptors in cell signalling

Objective: In this unit you will know about Cell signalling; its general principles, role of cell surface receptors in cell signalling.

Introduction

Cell signalling is the process of cells communicating with other cells within the body, or with the external environment. As a process, cell signalling refers to a vast network of communication between, and within, each cell of our body. With cell signalling, cells are able to coordinate within large, multicellular organisms.

Cell signalling can occur through a number of different pathways, but the overall theme is that the actions of one cell influence the function of another. Cell signalling is needed by multicellular organisms to coordinate a wide variety of functions. Nerve cells must communicate with muscle cells to create movement, immune cells must avoid destroying cells of the body, and cells must organize during the development of a baby.

Most cell signals are chemical in nature. For example, prokaryotic organisms have sensors that detect nutrients and help them navigate toward food sources. In multicellular organisms, growth factors, hormones, neurotransmitters, and extracellular matrix components are some of the many types of chemical signals cells use. These substances can exert their effects locally, or they might travel over long distances. For instance, neurotransmitters are a class of short-range signalling molecules that travel across the tiny spaces between adjacent neurons or between neurons and muscle cells. Other signalling molecules must move much farther to reach their targets. One example is follicle-stimulating hormone, which travels from the mammalian brain to the ovary, where it triggers egg release.

Some cells also respond to mechanical stimuli. For example, sensory cells in the skin respond to the pressure of touch, whereas similar cells in the ear react to the movement of sound waves. In addition, specialized cells in the human vascular system detect changes in blood pressure — information that the body uses to maintain a consistent cardiac load.

Some forms of cell signalling are intracellular, while others are intercellular. Intracellular signals are produced by the same cell that receives the signal. On the other hand, intercellular signals can travel all throughout the body. This allows certain glands within the body to produce signals which take action on many different tissues across the body. Cell signalling is how a tiny gland within the brain can react to external stimuli and coordinate a response. In response to stimuli like light, odours, or touch, the gland can, in turn, release a hormone which activates responses in diverse body systems to coordinate a response to a threat or opportunity.

Basic mechanism of cell signalling

Cells typically communicate using chemical signals. These chemical signals, which are proteins or other molecules produced by a sending cell, are often secreted from the cell and released into the extracellular space (Fig 1).

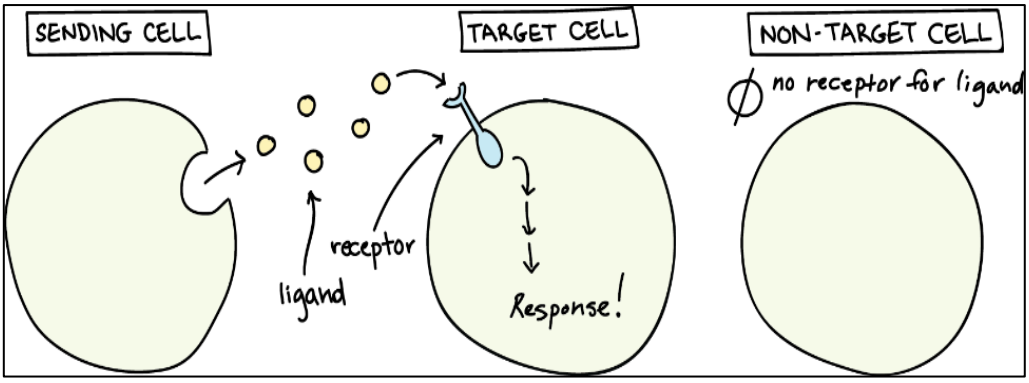


Fig 1: Basic mechanism of cell signalling; Sending cell: this cell secretes a ligand; Target cell: this cell has a receptor that can bind the ligand. The ligand binds to the receptor and triggers a signalling cascade inside the cell, leading to a response; Non-target cell: this cell does not have a receptor for the ligand (though it may have other kinds of receptors). The cell does not perceive the ligand and thus does not respond to it.

Not all cells can "hear" a particular chemical message. In order to detect a signal (that is, to be a target cell), a neighbour cell must have the right receptor for that signal. When a signalling molecule binds to its receptor, it alters the shape or activity of the receptor, triggering a change inside of the cell. Signalling molecules are often called ligands, a general term for molecules that bind specifically to other molecules (such as receptors). The message carried by a ligand is often relayed through a chain of chemical messengers inside the cell. Ultimately, it leads to a change in the cell, such as alteration in the activity of a gene or even the induction of a whole process, such as cell division. Thus, the original intercellular (between-cells) signal is converted into an intracellular (within-cell) signal that triggers a response.

Forms of signalling

Cell-cell signalling involves the transmission of a signal from a sending cell to a receiving cell. However, not all sending and receiving cells are next-door neighbours, nor do all cell pairs exchange signals in the same way.

There are four basic categories of chemical signalling found in multicellular organisms: paracrine signalling, autocrine signalling, endocrine signalling, and signalling by direct contact. The main difference between the different categories of signalling is the distance that the signal travels through the organism to reach the target cell.

1. Paracrine signalling

Often, cells that are near one another communicate through the release of chemical messengers (ligands that can diffuse through the space between the cells). This type of signalling, in which cells communicate over relatively short distances, is known as paracrine signalling.

Paracrine signalling allows cells to locally coordinate activities with their neighbours. Although they're used in many different tissues and contexts, paracrine signals are especially important during development, when they allow one group of cells to tell a neighbouring group of cells what cellular identity to take on. Example:

i) spinal cord development

Cross-section of the developing spinal cord, showing the distribution of Shh and the specification of different types of neurons. As Shh diffuses away from the notochord and floor plate, it forms a gradient, with high levels near the source and low levels further away (Fig 2). The different

concentrations of Shh at different points along the gradient help tell nearby cells what types of neurons they should become.

- Cells that are close to the notochord and floor plate receive a high dose of signal and become a specific type of connector neuron (interneuron).
- Cells that are a little further away get a lower dose of signal and become motor neurons (neurons that connect up to muscles).
- Cell that are even more distant from the notochord and floor plate receive progressively lower doses of signal and become other types of interneurons.

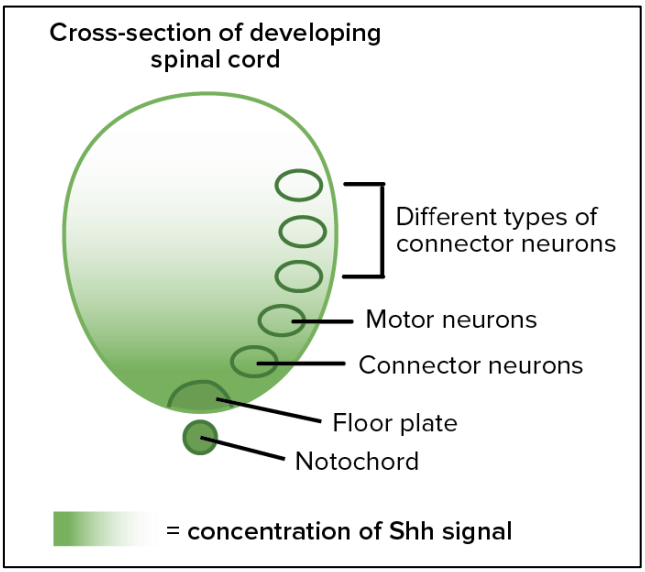


Fig 2: Paracrine signalling during spinal cord development

Different levels of Shh signal trigger different responses in cells, causing them to take on distinct identities and characteristics. Signals like Shh, which form gradients and produce different developmental effects depending on the dose of signal, are known as morphogens.

ii) Synaptic signalling

One unique example of paracrine signalling is synaptic signalling, in which nerve cells transmit signals (Fig 3). This process is named for the synapse, the junction between two nerve cells where signal transmission occurs.

When the sending neuron fires, an electrical impulse moves rapidly through the cell, traveling down a long, fibre-like extension called an axon. When the impulse reaches the synapse, it triggers the release of ligands called neurotransmitters, which quickly cross the small gap between the nerve cells. When the neurotransmitters arrive at the receiving cell, they bind to receptors and cause a chemical change inside of the cell (often, opening ion channels and changing the electrical potential across the membrane).

Neurotransmitter is released from vesicles at the end of the axon of the sending cell. It diffuses across the small gap between sending and target neurons and binds to receptors on the target neuron.

The neurotransmitters that are released into the chemical synapse are quickly degraded or taken back up by the sending cell. This "resets" the system so they synapse is prepared to respond quickly to the next signal.

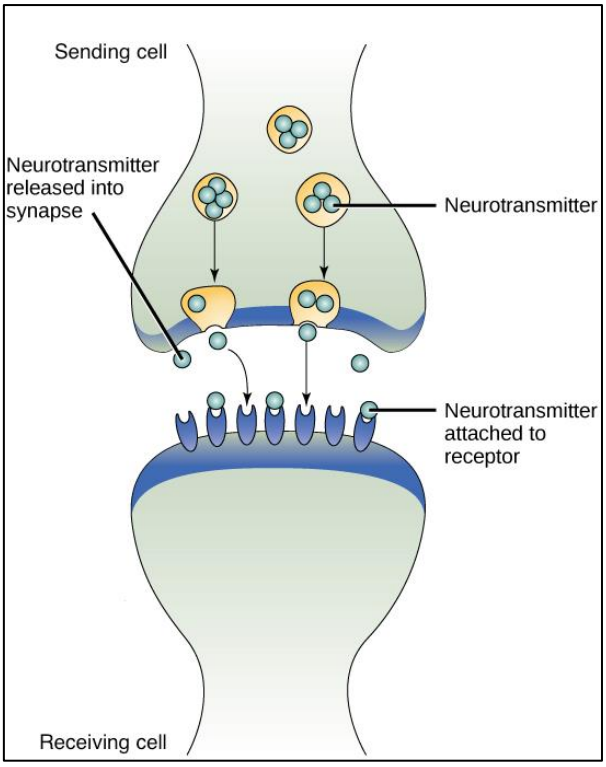


Fig 3: Synaptic signalling

2. Autocrine signalling

In autocrine signalling, a cell signals to itself, releasing a ligand that binds to receptors on its own surface (or, depending on the type of signal, to receptors inside of the cell). This may seem like an odd thing for a cell to do, but autocrine signalling plays an important role in many processes.

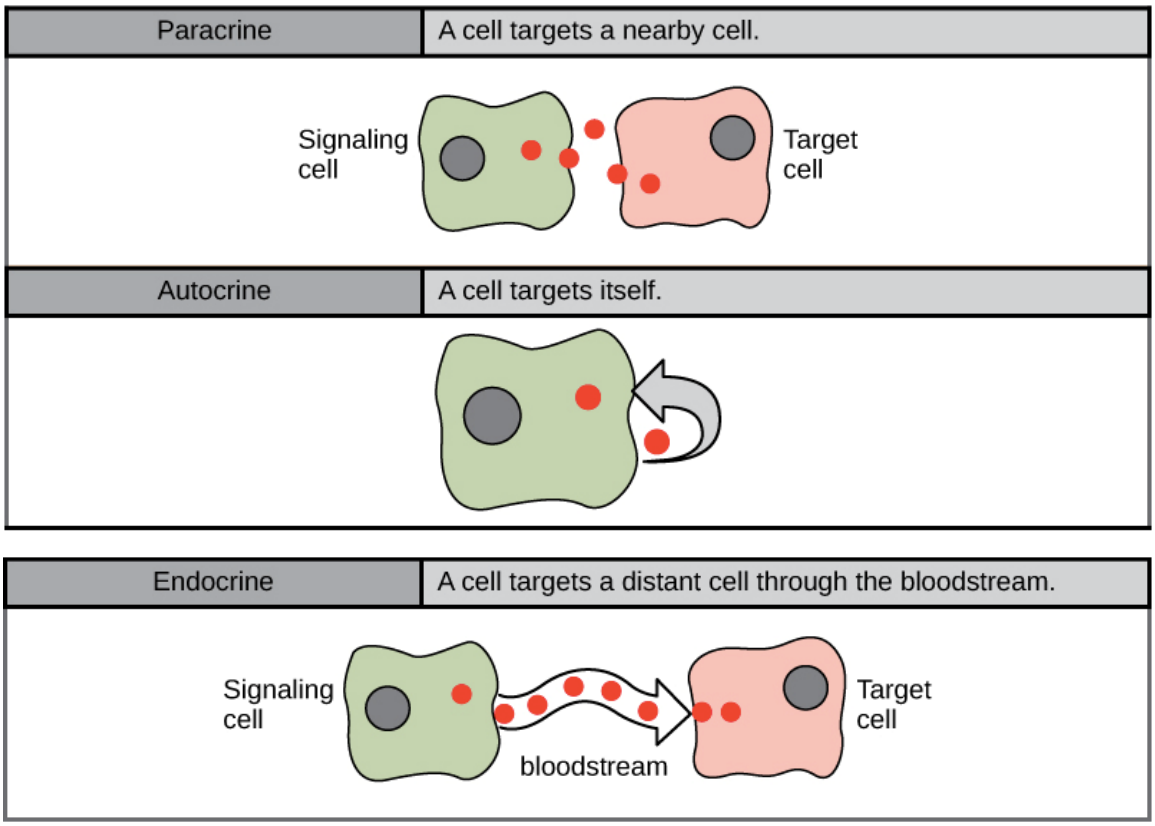
For instance, autocrine signalling is important during development, helping cells take on and reinforce their correct identities. From a medical standpoint, autocrine signalling is important in cancer and is thought to play a key role in metastasis (the spread of cancer from its original site to other parts of the body). In many cases, a signal may have both autocrine and paracrine effects, binding to the sending cell as well as other similar cells in the area.

3. Endocrine signalling

When cells need to transmit signals over long distances, they often use the circulatory system as a distribution network for the messages they send. In long-distance endocrine signalling, signals are produced by specialized cells and released into the bloodstream, which carries them to target cells in distant parts of the body. Signals that are produced in one part of the body and travel through the circulation to reach far-away targets are known as hormones.

In humans, endocrine glands that release hormones include the thyroid, the hypothalamus, and the pituitary, as well as the gonads (testes and ovaries) and the pancreas. Each endocrine gland releases one or more types of hormones, many of which are master regulators of development and physiology.

For example, the pituitary releases growth hormone (GH), which promotes growth, particularly of the skeleton and cartilage. Like most hormones, GH affects many different types of cells throughout the body. However, cartilage cells provide one example of how GH functions: it binds to receptors on the surface of these cells and encourages them to divide.



Paracrine signalling: a cell targets a nearby cell (one not attached by gap junctions). The image shows a signalling molecule produced by one cell diffusing a short distance to a neighbouring cell.

Autocrine signalling: a cell targets itself, releasing a signal that can bind to receptors on its own surface.

Endocrine signalling: a signalling molecule produced by one cell transmits over long distance using circulatory pathway.

Cell surface receptors

Cells have proteins called receptors that bind to signalling molecules and initiate a physiological response. Different receptors are specific for different molecules. Dopamine receptors bind dopamine, insulin receptors bind insulin, nerve growth factor receptors bind nerve growth factor, and so on. In fact, there are hundreds of receptor types found in cells, and varying cell types have different populations of receptors. Receptors can also respond directly to light or pressure, which makes cells sensitive to events in the atmosphere.

Receptors are generally transmembrane proteins, which bind to signalling molecules outside the cell and subsequently transmit the signal through a sequence of molecular switches to internal signalling pathways.

There are two types of receptors:

1. Internal receptors (intracellular or cytoplasmic receptors) and

2. cell-surface receptors.

1. Internal receptors

Internal receptors, also known as intracellular or cytoplasmic receptors, are found in the cytoplasm of the cell and respond to hydrophobic ligand molecules that are able to travel across the plasma membrane (Fig 4). Once inside the cell, many of these molecules bind to proteins that act as regulators of mRNA synthesis to mediate gene expression. Gene expression is the cellular process of transforming the information in a cell's DNA into a sequence of amino acids that ultimately forms a protein. When the ligand binds to the internal receptor, a conformational change exposes a DNA-binding site on the protein. The ligand-receptor complex moves into the nucleus, binds to specific regulatory regions of the chromosomal DNA, and promotes the initiation of transcription. Internal receptors can directly influence gene expression without having to pass the signal on to other receptors or messengers.

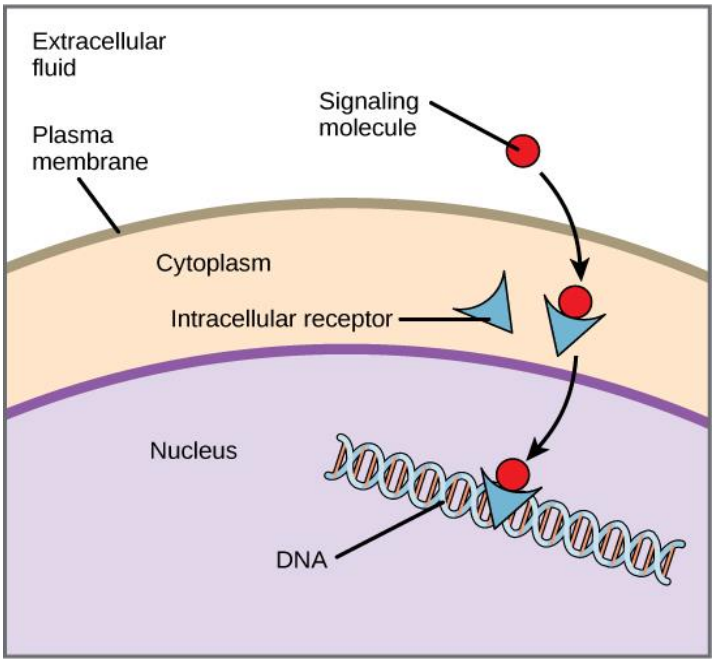


Fig 4: Intracellular Receptors: Hydrophobic signalling molecules typically diffuse across the plasma membrane and interact with intracellular receptors in the cytoplasm. Many intracellular receptors are transcription factors that interact with DNA in the nucleus and regulate gene expression.

2. Cell-Surface Receptors

Cell-surface receptors, also known as transmembrane receptors, are cell surface, membrane-anchored, or integral proteins that bind to external ligand molecules. This type of receptor spans the plasma membrane and performs signal transduction, converting an extracellular signal into an intracellular signal. Ligands that interact with cell-surface receptors do not have to enter the cell that they affect. Cell-surface receptors are also called cell-specific proteins or markers because they are specific to individual cell types.

Each cell-surface receptor has three main components: an external ligand-binding domain (extracellular domain), a hydrophobic membrane-spanning region, and an intracellular domain inside the cell. The size and extent of each of these domains vary widely, depending on the type of receptor.

Cell-surface receptors are involved in most of the signalling in multicellular organisms. There are three general categories of cell-surface receptors

I. G-protein-coupled receptors,

II. ion channel receptors, and

III. Enzyme-linked receptors.

I. G-protein-coupled receptors

G-Protein coupled receptors (GPCRs) are a group of seven transmembrane proteins which bind signal molecules outside the cell, transduce the signal into the cell and finally cause a cellular response (Fig 5). The GPCRs work with the help of a G-Protein which binds to the energy rich GTP.

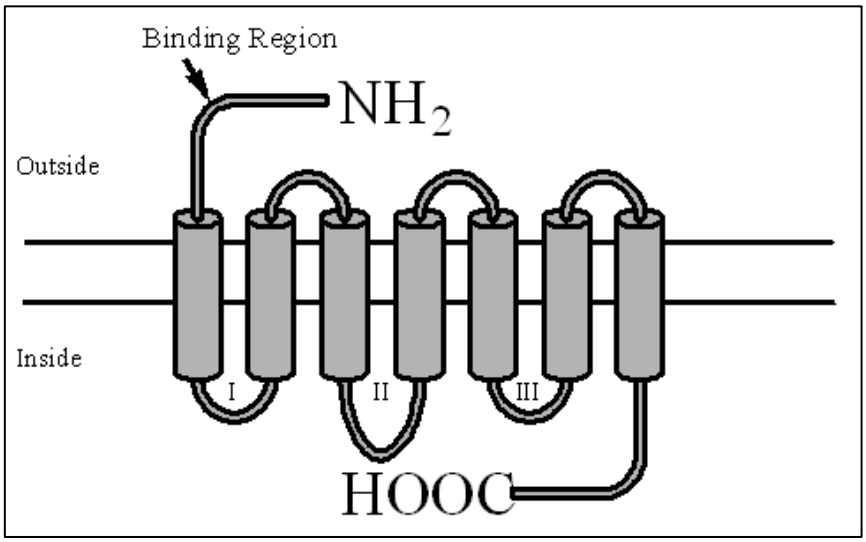


Fig 5: Seven transmembrane domain of G-protein coupled receptor

It is also known as heptahelical receptors, serpentine receptors, and G protein-linked receptors. These proteins make up transmembrane receptors whose purpose is to find molecules on the outside of the cell and initiate the signal transduction pathways. The signal transduction pathways are the processes by which a cell changes the form of one signal into the stimulus or a signal of another. These processes are carried out by enzymes. As the number of proteins and molecules increases, the size of the signal cascade increases rapidly, allowing for a large response, to a relatively small initiation factor.

G protein linked receptors are activated by ligands in the form of hormones, proteins, or other signalling molecule. This in turn leads to the activation of an intracellular G-protein by way of a certain interaction with the receptor. The G proteins act like relay batons to pass messages from circulating hormones into cells and transmit the signal throughout the cell with the ultimate goal of amplifying the signal in order to produce a cell response. Firstly, a hormone such as an epinephrine encounters a receptor in the membrane of a cell then a G protein is activated as it makes contact with the receptor to which the hormone is attached. Lastly, the G protein passes the message of a hormone to the cell by switching on a cell enzyme that triggers a response (Medicines by Design 46).

In addition to signalling, they have other physiological roles: -Sense of smell-the olfactory epithelium receptors bind odorants and pheromones -Mood regulation-receptors in the brain bind neurotransmitters (dopamine) -Immune system regulation- deals with inflammation and response to foreign bodies -Nervous system transmission-proteins control blood pressure, heart rate, and digestive processes -Cell density sensing.

G-Protein Coupled Receptor Structure

The structure of most G-Protein Coupled Receptors are not very well known. The typical method of determining protein structure is by x-ray crystallography of the protein once it has been crystallized. However, due to the membrane environment, flexibility, and dynamic shifting of GPCRs, it is difficult to form crystals of them. Some have been crystallized by mutating certain amino acids to stabilize the structure, but there is no universal way to study them all.

Although it is tough to find exact information about the structure there are a few known traits of the proteins. G-proteins represent the level of middle management in the cellular organisation and are able to communicate between the receptors and the effector enzymes or ion-channels. They were called G-proteins because of their interaction with the guanine nucleotides, GTP and GDP.

The G proteins are bound to the cytoplasmic surface of the plasma membrane. They are heterotrimeric molecules consisting of 3 subunits α, β and γ. Their classification as stimulatory or inhibitory is based on the identity of their distinct α subunit. The β and γ subunits remain associated as β γ complex with the cytoplasmic surface of the membrane when the system is inactive or in resting state, GDP is bound to the α subunit.

Conformational change

The receptor molecule exists in equilibrium between the active and inactive states (Fig 6). The ligand binding pushes the equilibrium towards the active sites. There are three types of ligands that bind to the g-proteins. The first are agonists, ligands that shift the equilibrium towards the active states. Inverse agonist shift the equilibrium towards the inactive states, and neutral antagonists are ligands that do not change the equilibrium.

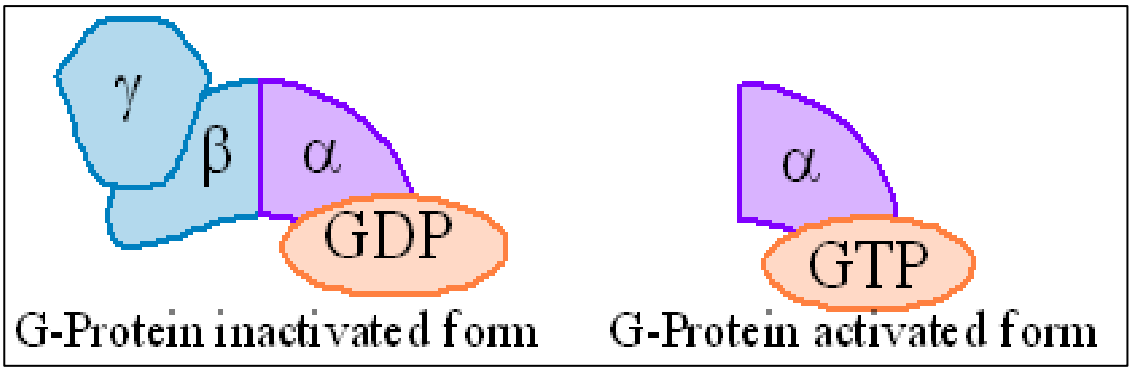


Fig 6: Active and inactive state of G-protein coupled receptor

Mechanism of G-protein coupled receptor

Cell signalling using G-protein-linked receptors occurs as a cyclic series of events (Fig 7). Before the ligand binds, the inactive G-protein can bind to a newly revealed site on the receptor specific for its binding. Once the G-protein binds to the receptor, the resultant shape change activates the G-protein, which releases GDP and picks up GTP. The subunits of the G-protein then split into the α subunit and the βγ subunit. One or both of these G-protein fragments may be able to activate other proteins as a result. After a while, the GTP on the active α subunit of the G-protein is hydrolysed to GDP and the βγ subunit is deactivated. The subunits reassociate to form the inactive G-protein and the cycle begins a new.

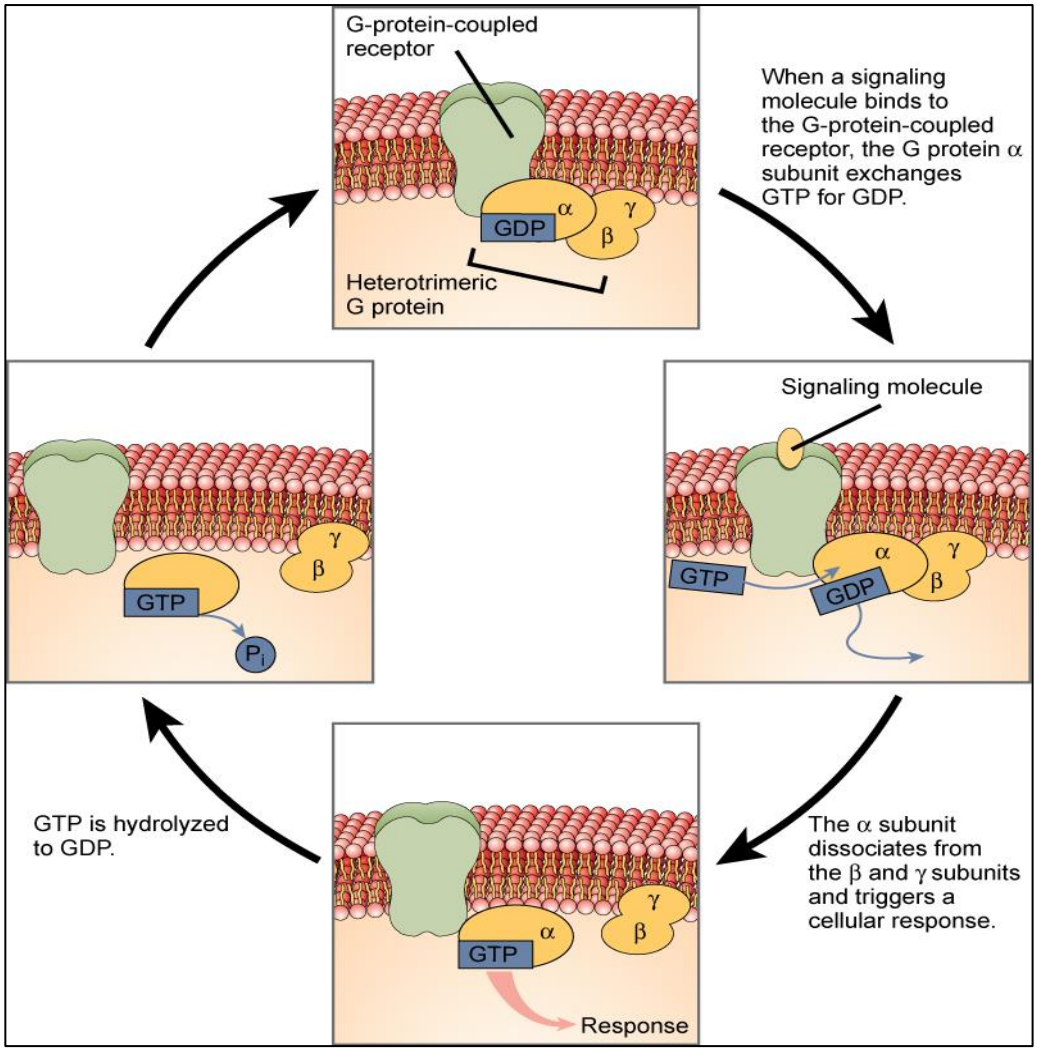


Fig 7: Heterotrimeric G proteins have three subunits: α, β, and γ. When a signalling molecule binds to a G-protein-coupled receptor in the plasma membrane, a GDP molecule associated with the α subunit is exchanged for GTP. The β and γ subunits dissociate from the α subunit, and a cellular response is triggered either by the α subunit or the dissociated $\beta\gamma$ pair. Hydrolysis of GTP to GDP terminates the signal.

Uses of G-Protein Coupled Receptors

G-Protein Coupled Receptors are widespread in their use. In the eyes, Opsins, a GPCRs, translate electromagnetic radiation into cellular signals thus allowing visual perception. In the nose, olfactory epithelium binds odorants and pheromones which allow for the sense of smell. However, there are problems associated with GPCRs. Many human diseases, including bacterial infections, involve the GPCRs where bacteria produce toxins which interfere with the function of the G-Protein. Examples of such diseases include cholera, whooping cough, botulism, etc.

Here is a closer view of how altered G-protein affects cholera and whooping cough. When there is a β subunit binding to $G_{\alpha s}$ gangliosides and a catalytic subunit entering the cell, then choleragen, a toxin resulted from cholera, forms. The catalytic subunit alters the $G_{\alpha s}$ ganglioside in which α part of the protein is adjusted via attaching ADP-ribose to arginine. As a result of this alteration, $G_{\alpha s}$ ganglioside become stable, meaning that now $G_{\alpha s}$ ganglioside is in its active form. The active $G_{\alpha s}$ ganglioside then does its job to activate protein kinase A, denoted PKA. The chloride channel is by PKA, PKA enters

and thus there is no more absorption of Na^+. This is saying that there is a huge loss of NaCl and water in the body as seen in the symptoms of cholera. In treating cholera, the most effective way is to rehydrate the body using glucose-electrolyte solution.

Whooping cough, on the other hand, is different from cholera in which the ADP ribose moiety is added by the toxin. In this case, Ca^{2+} channel is closed whereas the K^+ channel is opened by $G_{\alpha s}$ ganglioside. The result is that the $G_{\alpha s}$ ganglioside is in its inactive form and ultimately ending up with uncontrollable coughs.

In addition, Pharmacologists estimate that 60% of all medicines achieve their effect by acting on G Protein pathways. Since G protein is a switch molecule which passes the message inward (like relay baton), it can be turned on only when needed, then shut off. Some illnesses like cholera, occur when a G protein is errantly left on. Discovery about G protein switches and its structure which is made into 3 subunits (α, β, γ) will help us understand how we can inhibit the transmission or increase with some other ligand. G-protein coupled receptors are trans-membrane receptor proteins that when activated by ligands (hormones, proteins or other signalling molecules), they lead to the activation of an intracellular G-protein through a specific interaction with the receptor. The G-protein in turn transmits the signal to other proteins within the cell to ultimately amplify the signal and produce a cellular response. Understanding the structure and dynamics of the receptor could clarify the specific interactions the receptor makes with the ligand on the outside and the G-protein on the intracellular side, thereby leading to the understanding of how the receptor works. Consequently, drugs (agonists or antagonists) can be designed to bind the receptor and control its response, whether it's to transmit the signal to the G-protein or inhibit the transduction. Moreover, monitoring the effects of such signals can help in understanding the type of induced cellular responses and potentially uncover diseases that are proliferated in this manner.

II. Ion channel receptors

Ion channels are membrane protein complexes and their function is to facilitate the diffusion of ions across biological membranes. Membranes, or phospholipid bilayers, build a hydrophobic, low dielectric barrier to hydrophilic and charged molecules. They are electrical insulators. Ion channels provide a high conducting, hydrophilic pathway across the hydrophobic interior of the membrane. The channel, or pore structure, is said to catalyse the 'reaction' of transporting charged molecules across a low dielectric medium. The 'catalytic site', the central channel, is either open or closed. The open channel conformation can be compared to the transition state of the enzyme-substrate complex, where ions are tightly associated to the catalytic site. The conformational change between closed and open state is called gating, as in opening and closing a gate. Channel gating is controlled by external factors like enzymes are controlled by modulators and effectors.

Ion channels can be classified according to which chemical or physical modulator controls their gating activity. There are three main types of ion channels, i.e., voltage-gated, extracellular ligand-gated, and intracellular ligand-gated along with two groups of miscellaneous ion channels.

Mechanism of ligand-gated ion channels

Chemical signals are released by signalling cells in the form of small, usually volatile or soluble molecules called ligands. A ligand is a molecule that binds another specific molecule, in some cases, delivering a signal in the process. Ligands can thus be thought of as signalling molecules. Ligands interact with proteins in target cells, which are cells that are affected by chemical signals; these proteins are also called receptors. Ligands and receptors exist in several varieties; however, a specific ligand will have a specific receptor that typically binds only that ligand.

When a ligand binds to the extracellular region of the channel, the protein's structure changes in such a way that ions of a particular type, such as Ca^{+2} or Cl^{-1} can pass through (Fig 8). In some cases, the reverse is actually true: the channel is usually open, and ligand binding causes it to close. Changes in ion levels inside the cell can change the activity of other molecules, such as ion-binding enzymes and voltage-sensitive channels, to produce a response. Neurons, or nerve cells, have ligand-gated channels that are bound by neurotransmitters.

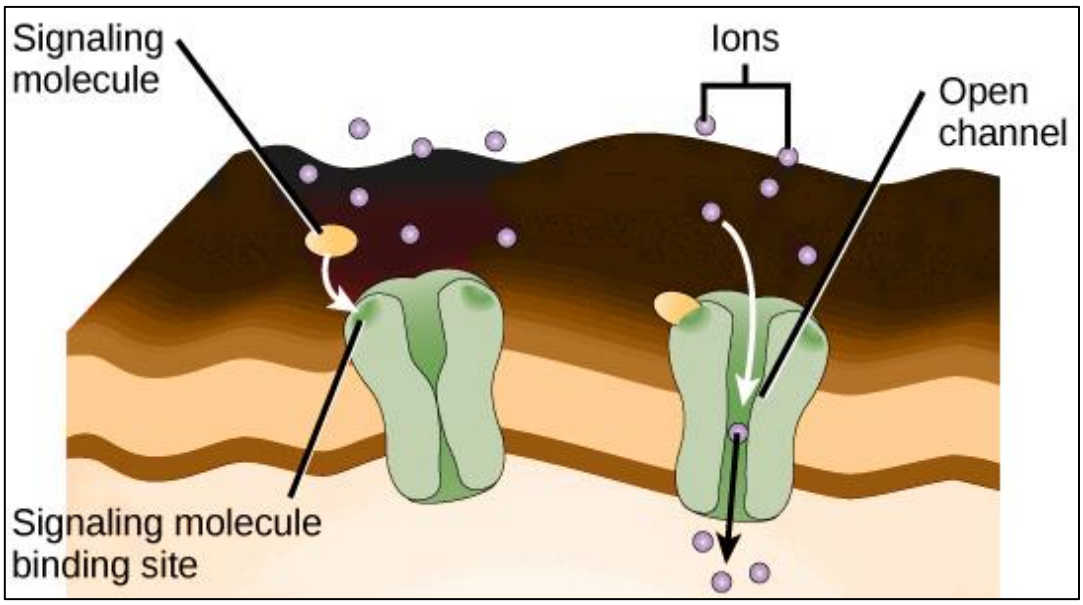


Fig 8: Gated ion channels form a pore through the plasma membrane that opens when the signalling molecule binds. The open pore then allows ions to flow into or out of the cell.

Voltage-gated ion channels

Voltage-gated ion channels are generally composed of several subunits arranged in such a way that there is a central pore through which ions can travel down their electrochemical gradients. The channels tend to be ion-specific, although similarly sized and charged ions may sometimes travel through them. The functionality of voltage-gated ion channels is attributed to its three main discrete units: the voltage sensor, the pore or conducting pathway, and the gate (Fig 9). Na^+, K^+, and Ca^{2+} channels are composed of four transmembrane domains arranged around a central pore; these four domains are part of a single α-subunit in the case of most Na^+ and Ca^{2+} channels, whereas there are four α-subunits, each contributing one transmembrane domain, in most K^+ channels. The membrane-spanning segments, designated S1-S6, all take the form of alpha helices with specialized functions. The fifth and sixth transmembrane segments (S5 and S6) and pore loop serve the principal role of ion conduction, comprising the gate and pore of the channel, while S1-S4 serve as the voltage-sensing region. The four subunits may be identical, or different from one another. In addition to the four central α-subunits, there are also regulatory β-subunits, with oxidoreductase activity, which are located on the inner surface of the cell membrane and do not cross the membrane, and which are co-assembled with the α-subunits in the endoplasmic reticulum.

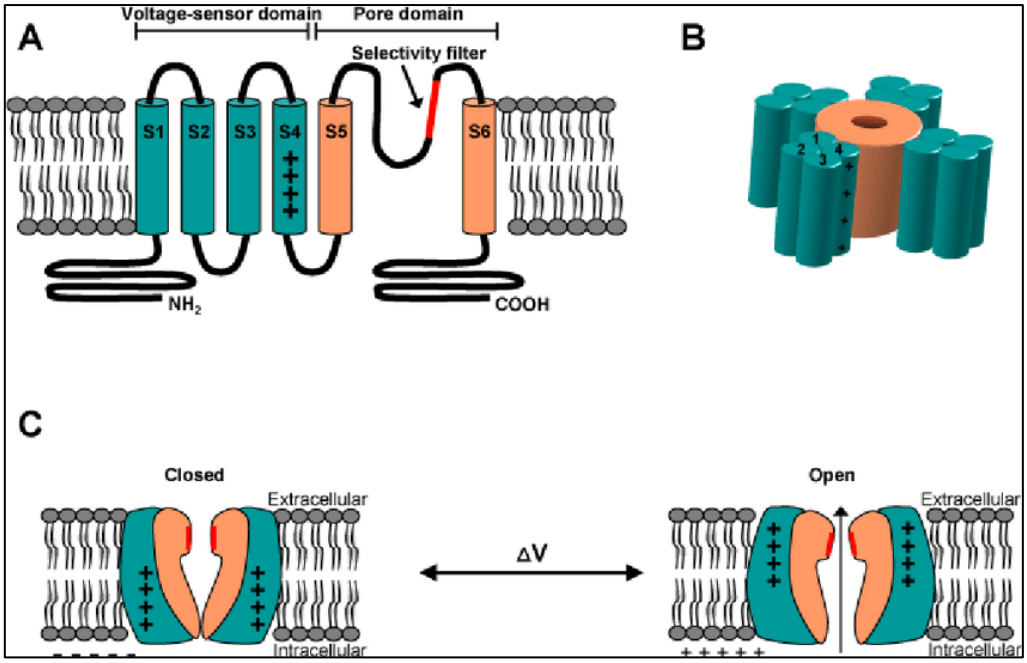


Fig 9: Structure of Voltage gated ion channel

Voltage-gated channels respond to perturbations in cell membrane potential, and are highly selective for a specific ion, i.e., Na^+, K^+, Ca^{2+}, and Cl^-. They are further subdivided into families based on the major permeant ion.

- *Voltage-gated Na^+ channel* - These channels are responsible for the generation of action potentials of long duration, and thus are targets of local anesthetics, such as lidocaine and benzocaine.
- *Voltage-gated Ca^{2+} channels* – They regulate intracellular Ca2+ concentrations, and thus are responsible for a wide range of biochemical processes within cells. One of the most important processes regulated by these channels is the release of neurotransmitters at synapses. Calcium channel blockers are valuable in treating a variety of conditions ranging from heart disease to anxiety disorders.
- *Voltage-gated K^+ channels* – They constitute the largest and the most diverse class of voltage-gated ion channels. They are imperative in generating the resting membrane potential.
- *Voltage-gated Cl^- channels* - These channels are present in every type of neuron and are involved in regulating excitability and cell volume. They are also known to contribute to the resting membrane potential.

Mechanism of Voltage-gated channels

Voltage-gated ion channels contain intrinsic voltage sensors. Voltage-gated ion channels typically are closed at the resting membrane potential but open upon membrane depolarization. These channels detect changes in electric potential across the membrane through a domain responsible for sensing voltage. The voltage sensor spans the membrane and is thus exposed to the electric field across the phospholipid bilayer. Charged residues in this sensor move in response to changes in membrane potential and trigger conformational changes of the channel.

Voltage-gated Na+ channels have three main conformational states: closed, open and inactivated. Forward/back transitions between these states are correspondingly referred to as

activation/deactivation (between open and closed, respectively), inactivation/reactivation (between inactivated and open, respectively), and recovery from inactivation/closed-state inactivation (between inactivated and closed, respectively). Closed and inactivated states are ion impermeable.

Before an action potential occurs, the axonal membrane is at its normal resting potential, and Na+ channels are in their deactivated state, blocked on the extracellular side by their activation gates. In response to an increase of the membrane potential to about -55 mv (in this case, caused by an action potential), the activation gates open, allowing positively charged Na+ ions to flow into the neuron through the channels, and causing the voltage across the neuronal membrane to increase to +30 mv in human neurons. Because the voltage across the membrane is initially negative, as its voltage increases to and past zero (from -70 mv at rest to a maximum of +30 mv), it is said to depolarize. This increase in voltage constitutes the rising phase of an action potential.

At the peak of the action potential, when enough Na^+ has entered the neuron and the membrane's potential has become high enough, the Na^+ channels inactivate themselves by closing their inactivation gates. The inactivation gate can be thought of as a "plug" tethered to domains III and IV of the channel's intracellular alpha subunit. Closure of the inactivation gate causes Na^+ flow through the channel to stop, which in turn causes the membrane potential to stop rising. With its inactivation gate closed, the channel is said to be inactivated. With the Na^+ channel no longer contributing to the membrane potential, the potential decreases back to its resting potential as the neuron repolarizes and subsequently hyperpolarizes itself. This decrease in voltage constitutes the falling phase of the action potential.

When the membrane's voltage becomes low enough, the inactivation gate reopens and the activation gate closes in a process called deactivation. With the activation gate closed and the inactivation gate open, the Na^+ channel is once again in its deactivated state, and is ready to participate in another action potential.

When any kind of ion channel does not inactivate itself, it is said to be persistently (or tonically) active. Some kinds of ion channels are naturally persistently active. However, genetic mutations that cause persistent activity in other channels can cause disease by creating excessive activity of certain kinds of neurons. Mutations that interfere with Na^+ channel inactivation can contribute to cardiovascular diseases or epileptic seizures by window currents, which can cause muscle and/or nerve cells to become over-excited.

Uses of ion channels

The primary role of ion channels is to regulate the passage of ions across the cell membrane, and the alteration of intracellular ion concentration can be seen as the primary signaling mechanism of ion channels.

Ion channels are selective meaning that they only allow certain ions to pass through them, and they play critical roles in controlling neuronal excitability.

III. Enzyme-linked receptors

Enzyme-linked receptors are cell-surface receptors with intracellular domains that are associated with an enzyme. In some cases, the intracellular domain of the receptor actually *is* an enzyme that can catalyze a reaction. Other enzyme-linked receptors have an intracellular domain that interacts with an enzyme. They were recognized initially through their role in responses to extracellular signal proteins that promote the growth, proliferation, differentiation, or survival of cells in animal tissues.

Classification

There are five main types of enzyme-linked receptors:

1. Receptor Tyrosine Kinase (RTK): Contains intrinsic tyrosine kinase activity (EGFR, VEGFR)
2. Receptor Serine/Threonine Kinase: Contains intrinsic serine/threonine kinase activity (TGF-βR)
3. Receptor Guanylyl Cyclases: Contain intrinsic cyclase activity (ANP)
4. Tyrosine-Kinase Associated Receptors: Receptors that associate with proteins that have tyrosine kinase activity (Cytokine Receptors)
5. Receptor Tyrosine Phosphatases

Receptor tyrosine kinases (RTKs) are a class of enzyme-linked receptors found in humans and many other species. A kinase is just a name for an enzyme that transfers phosphate groups to a protein or other target, and a receptor tyrosine kinase transfers phosphate groups specifically to the amino acid tyrosine.

Mechanism of Receptor tyrosine kinases (RTKs) activity

Signalling molecules first bind to the extracellular domains of two nearby receptor tyrosine kinases (Fig 10).

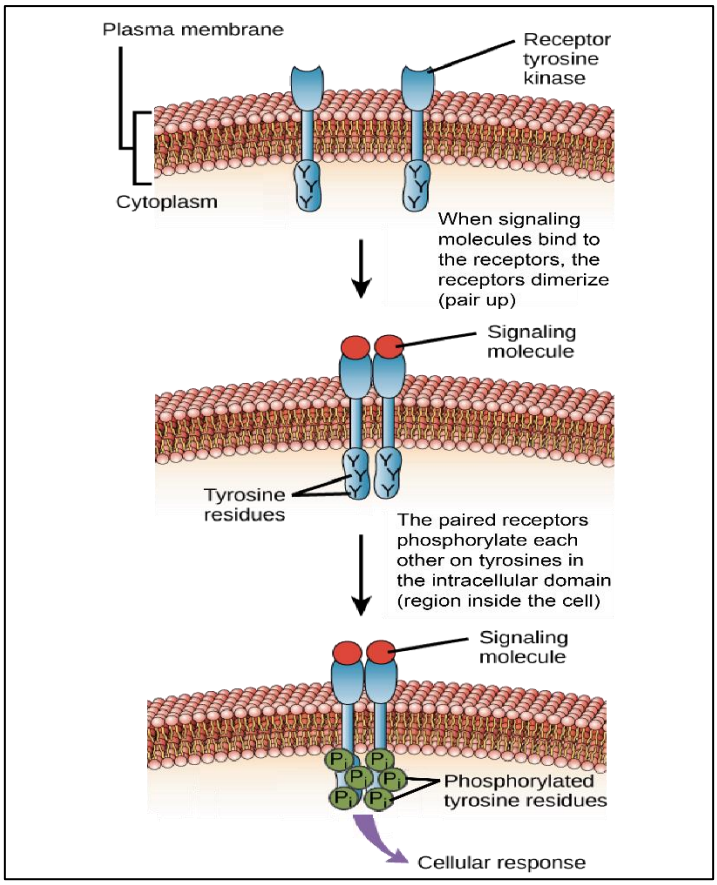


Fig 10: A receptor tyrosine kinase is an enzyme-linked receptor with a single transmembrane region, and extracellular and intracellular domains. Binding of a signalling molecule to the extracellular domain causes the receptor to dimerize. Tyrosine residues on the intracellular domain are then auto phosphorylated, triggering a downstream cellular response. The signal is terminated by a phosphatase that removes the phosphates from the phosphotyrosine residues.

The two neighbouring receptors then come together, or dimerize. The receptors then attach phosphates to tyrosines in each other's' intracellular domains. The phosphorylated tyrosine can transmit the signal to other molecules in the cell.

In many cases, the phosphorylated receptors serve as a docking platform for other proteins that contain special types of binding domains. A variety of proteins contain these domains, and when one of these proteins binds, it can initiate a downstream signalling cascade that leads to a cellular response. Receptor tyrosine kinases are crucial to many signalling processes in humans. For instance, they bind to growth factors, signalling molecules that promote cell division and survival. Growth factors include platelet-derived growth factor (PDGF), which participates in wound healing, and nerve growth factor (NGF), which must be continually supplied to certain types of neurons to keep them alive. Because of their role in growth factor signalling, receptor tyrosine kinases are essential in the body, but their activity must be kept in balance: overactive growth factor receptors are associated with some types of cancers.

Uses of Enzyme-linked receptors

Kinase is a large family of enzymes that are responsible for catalysing the transfer of a phosphoryl group from a nucleoside triphosphate donor, such as ATP, to an acceptor molecule. Tyrosine kinases catalyse the phosphorylation of tyrosine residues in proteins. The phosphorylation of tyrosine residues in turn causes a change in the function of the protein that they are contained in.

Phosphorylation at tyrosine residues controls a wide range of properties in proteins such as enzyme activity, subcellular localization, and interaction between molecules. Furthermore, tyrosine kinases function in many signal transduction cascades wherein extracellular signals are transmitted through the cell membrane to the cytoplasm and often to the nucleus, where gene expression may be modified. Finally mutations can cause some tyrosine kinases to become constitutively active, a nonstop functional state that may contribute to initiation or progression of cancer.

The receptor tyrosine kinases function in transmembrane signalling, whereas tyrosine kinases within the cell function in signal transduction to the nucleus. Tyrosine kinase activity in the nucleus involves cell-cycle control and properties of transcription factors.

Probable questions:

1. Define cell signalling.
2. Describe the basic mechanism of cell signalling with diagram.
3. What do you mean by paracrine signalling? Give example.
4. Write short note on G-protein-linked receptors.
5. Define Ion-gated channel. Describe the mechanism of ligand-gated ion channels.
6. Describe the mechanism of Receptor tyrosine kinases

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Unit III

Cancer: Characteristics of tumour cells; Oncogenes and their proteins

Objective: In this unit you will know about basic idea of cancer, characteristics of tumour cells, oncogenes and their proteins.

Introduction

In multicellular organisms, cell division is a normal process. Cells divide for growth, for the development of organs, for healing of wounds and also for the replacement of older and damaged cells. Cell division is a very complex process which is controlled by a regulatory mechanism at both molecular and cellular level. In higher multicellular organism, each and every cell belongs to a particular type of tissue like epithelial tissue, connective tissue muscular tissue etc. Hence, when a cell of a specific tissue divides, it normally produces its own kinds of cell of the tissue to which it belongs. It never produces the cells of other tissues. Therefore, the process by which cells achieve this specification and specialisation is known as cellular differentiation. Differentiation of cell begins during embryonic gastrulation stage and continues through tissue formation. Actually differentiation has a genetic basis and the process results from the interaction of the nucleus and the cytoplasm. After the cells become well differentiated, they cannot go back normally to the undifferentiated stage unless disturbed internally or externally.

Therefore, in multicel-lular organism, the cell division, differentiation and survival of individual cells are carefully regulated to meet the needs of the organism as a whole. When this regulation is lost due to any reason, the cells behave unusually and defy their control mechanism. Then the cells grow and divide in an uncontrolled manner ultimately spreading throughout the body and interfering with the functions of normal tissues and organs. As a whole, this condition leads to cancer. Cancer develops from defects in fundamental regulatory mechanisms of the cell.

Cancer is a non-infectious disease. It starts at the molecular level of the cell and, ultimately affects the cellular behaviour. Generally, it can be defined as uncontrolled proliferation of cells without any differentiation. Cancer cells are different from normal cells in some aspects. They do not remain confined to one part of the body. They penetrate and infiltrate into the adjoining tissues and dislocate their functions. Some of the cancer cells get detached from the main site of origin and travel by blood and lymph to sites distant from the original tumour and form fresh colonies, called metastasis or secondary growth.

Types of Cancer:

Cancer is a large class of diverse disease. All types of cancer can result from uncontrolled cell growth and division of any of the different kinds of cells in the body. So there are more than a hundred distinct types of cancer which vary in their behaviour and response to treatment.

The uncontrolled cell growth produces a mass of cells which are called tumours or neoplasm tumours may be benign or malignant. A benign tumour remains confined to its original location. They do not invade the surrounding normal tissues. They do not spread to distant body sites.

The most common example of tumour is the skin wart. A benign tumour consists of closely resembles normal cells and may function like normal cells. Generally benign tumours are harmless and can usually be removed surgically. However, these tumours may sometimes become quite harmful if they are located in organs like brain and liver.

A malignant tumour does not remain confined to its original location. They are capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems. Malignant tumours become life-threatening if, they spread throughout the body.

Only malignant tumours are properly designated as cancers. The cells of malignant tumour are derived from single cell, thus they are monoclonal in character. Malignant tumour is composed of aberrant cells. They behave like embryonic type, undifferentiated, having irregular, large nucleus, and deficient of cytoplasm. Malignant tumours are generally classified into four main types on the basis of cell type from which they arise.

(i) Carcinomas:

It includes approximately 90% of human cancer. This type is principally derived from epithelial cells of ectoderm and endoderm. The solid tumours in nerve tissue and in tissues of body surfaces or their attached glands are example of carcinomas. Cervical, breast, skin and brain carcinomas are developed from malignant tumour.

(ii) Sarcomas:

These cancers are located in connective and muscular tissues derived from mesoderm. Thus, they include the cancers of bones, cartilages, tendons, adipose tissue, lymphoid tissue and muscles.

(i) Cancer of bones is called osteoma.

(ii) Cancers of adipose tissue are known as lipomas.

(iii) Cancer of lymphoid tissues is called lymphoma. Hodgkin's disease is an example of human lymphoma. In Hodgkin's disease there is chronic enlargement of the production of lymphocytes by lymph nodes and spleen. They are rare in humans; about 1 per cent of all tumours are sarcomas.

(iii) Lymphomas:

It is a type of malignancy in which there is excessive production of lymphocytes by the lymph nodes and spleen. It accounts for approximately 8% of human cancers. Hodgkin's disease is an example of human lymphoma.

(iv) Leukaemia:

This type of malignancy arises from the blood forming cell. Leukaemias are commonly known as blood cancer. Leukaemia are neoplastic growth (uncontrolled cell growth at the cost of remaining cells) of leucocytes or WBC.

They are characterised by excessive production of WBC of the blood. The name leukaemia is derived from Greek leukos (white) + haima (blood) the massive proliferation of leukaemia cells can cause a patient's blood to appear milky.

In addition to the types of cancer mentioned above, cancers are further classified according to tissue of origin, for example lung cancer, breast cancer, and the type of cells involved, for example fibro sarcoma arises from fibroblasts, erythromoid leukemia's from precursor of erythrocytes. Although there are many kinds of cancer, the four most common cancers are those of prostrate, breast, lung and colon/rectum.

Characteristics of tumour cells

All types of cancer can result from uncontrolled cell growth and division of any of the different kinds of cells in the body. The uncontrolled cell growth produces a mass of cells which are called tumours or neoplasm tumours may be benign or malignant.

Types of Tumours

There are two types of tumours: *benign* and *malignant*.

(i) Benign Tumour (= Non-malignant Tumour):

A benign tumour remains confined to its original location. They do not invade the surrounding normal tissues. They do not spread to distant body sites. It causes limited damage to the body. It is non-cancerous.

The most common example of tumour is the skin wart. A benign tumour consists of closely resembles normal cells and may function like normal cells. Generally benign tumours are harmless and can usually be removed surgically. However, these tumours may sometimes become quite harmful if they are located in organs like brain and liver.

(ii) Malignant Tumour (= Cancerous Tumour):

A malignant tumour does not remain confined to its original location. It first grows slowly. No symptoms are noticed. This stage is called the latent stage. The tumour later grows quickly. The cancer cells go beyond adjacent tissue and enter the blood and lymph. Once this happens, they migrate too many other sites in the body where the cancer cells continue to divide. A phenomenon in which cancer cells spread to distant sites through body fluids to develop secondary tumour is called metastasis. Only malignant tumours are properly designated as cancer.

Properties of Cancer Cells,

(i) Uncontrolled proliferative ability,

(ii) Extracellular growth factors are not required,

(iii) Overgrowth and ability to invade new sites (metastasis),

(iv) Nucleus becomes irregular with abundant granules,

(v) There is increase in number of lysosomes, reduction in mitochondrial cristae, more melanin and debris in cytoplasm,

(vi) Cancer cells resist induction of cell death which promotes development of tumours.

Characteristics of Cancer Cells/ Malignant tumour cells

The uncontrolled growth of cancer cells results from accumulated abnormalities affecting many of the cell regulatory mechanisms. The process of cell change in which a normal cell loses its ability to control its rate of division and thus becomes a tumour cell is called cell transformation.

Cancer cells shows some typical characteristic properties that are absent in normal cells. Sometimes cancer cell properties are just opposite to the properties of normal cell. Cancer cells in vivo differ from their normal counterparts in several respects. Some characteristic properties of cancer cells can also be demonstrated by cell culture in vitro.

(i) Immortalization:

Normal cell culture do not survive indefinitely for example, human cell culture die after about 50 generations. On the other hand, transformed cell cultures can go on indefinitely and remain immortal if the nutrition is provided and overcrowding avoided.

(ii) Loss of Contact Inhibition:

Normal cells growing in tissue culture tend to make cell contacts by adhesion to neighbouring cells. At the points of adhesion some kind of electron-dense plaque is formed in both contacting cells. At the same time there is a slowing down of the amoeboid process which results in contact inhibition of movement. In contrast, cancer cells are unable to form adhesive junctions and do not show this type of contact inhibition.

Experimentally, it has been observed that when normal cells have become completely surrounded by other cells, their mobility stops and they form a monolayer. At the same time there is inhibition of growth and the number of cells in the petridish remains practically constant. On the other hand, cancer cells continue to multiply and pile up forming irregular masses several layers deep. Cancerous cells undergo a change in property of their cell membranes and cell coat such as disappearance of gap junction, loss of coupling changes in glycolipid and glycoprotein and a reduction in gangliosides.

In the cell coat fibronectin, a large glycoprotein found in footprints of moving cultured cells is reduced in cancerous cells. These changes enable the cells to dissociate from neighbouring cells and show loss of contact inhibition.

(iii) Reduced Cellular Adhesion:

Most cancer cells are less adhesive than the normal cells due to reduced expression of cell surface adhesive molecules. When normal cells are transformed into cancer cells, then a change of stickiness of their cell membrane results. Normal cells show stickiness or adhesiveness.

If normal cells are grown in a liquid nutrient medium kept in a glass vessel, the cells stick to glass wall rather than float in the medium. But when cancer cells are allowed to grow in nutrient medium, they stick to each other less than do normal cells.

Adhesiveness shows considerable specificity. For example, a liver cell tends to stick with another liver cell and not to other types of cell such as kidney cell. Cancerous cells do not show this property. They are able to mix and stick to any type of normal cell. For example, a malignant liver cell can mix and stick to normal kidney cell. Hence this unusual behaviour of cancer cell explains that cancer cells can invade several normal organs.

(iv) Invasiveness:

One of the most important characteristics of cancer cells is their invasiveness. It is the ability to invade other tissues. Malignant cells generally secrete proteases that digest extracellular matrix components, allowing the cancer cells to invade adjacent normal tissues. For example, secretion of collagenase by the cancer cells helps to digest and penetrate through basal laminae to invade the underlying connective tissue. Cancer cells also secrete growth factors that promote the formation of new blood vessels. This is known as angiogenesis. Angiogenesis is necessary to support the growth of tumour beyond the size of about a million cells at which point new blood vessels are needed to supply oxygen and nutrients to the multiplying tumour cells. Actually the growth factor secreted by the tumour cells stimulates the endothelial cells present in the wall of capillaries.

As a result, new outgrowth of the capillaries is formed into the tumour. These outgrowths of capillaries are also helpful for metastasis of malignant cells. Therefore, angiogenic stimulation induces the growth of new blood capillaries which penetrate easily in the tumour tissue and provide the opportunity for the cancer cells to enter the circulatory system. As a result, metastasis process begins.

(v) Failure to Differentiate:

Another general characteristic of most of the cancer cells is that they fail to differentiate. This property is closely related with the abnormal proliferation. Normal cells are fully differentiated. In most fully differentiated cells, cell division ceases. In case of cancer-cells, normal differentiation program is blocked at the early stages of differentiation. The relationship between defective differentiation and rapid proliferation is clearly noted in case of leukaemia.

All of the different types of blood cells develop from a common pluripotent stem cell in the bone marrow. Some of the descended cells develop erythrocytes but others differentiate to form lymphocytes, granulocytes and macrophages. Cells of each of these types become round as they differentiate but once they become fully differentiated cell division ceases But leukaemia cells fail to undergo terminal differentiation. Instead, they become blocked at early stage of maturation at which they retain their capacity for proliferation and continue to divide.

(vi) Auto stimulation of Cell Division:

Cancer cells produce growth factor that stimulates their own cell division. Such abnormal production of a growth factor by the cancer cell leads to continuous auto stimulation of cell division. This is known as autocrine growth stimulation. Hence the cancer cells are less dependent on general growth factor produced within the body physiologically from normal source for inducing growth of all normal cells. It is also noted that the reduced growth factor dependence of cancer cell results from abnormalities in intracellular signalling system.

(vii) Apoptosis:

For every cell, there is a fixed span of life, i.e., time to live and time to die. This cell death is a very orderly process and so it is called Programmed Cell Death or PCD or Apoptosis. Apoptosis is a mechanism of programmed cell death or cell suicide which is essential for the survival of the organism, for the normal development of the organism as the programmed destruction of the organism as the programmed destruction of cells is found during embryo-genesis. It also protects the organism by removing damaged cells which may be due to viral infection or due to exposure to radiations. It also inhibits the tumour development and so any defect in the control of apoptosis may lead to cancer.

Difference between cancer cells and normal cells

These ‘normal’ cells act as the body’s basic building blocks and possess specific characteristics that enable them to maintain correct functioning of tissues, organs, and organ systems. Normal cells:

- control their growth using external signals, meaning they only grow and divide when required,
- undergo programmed cell death (apoptosis) as part of normal development, to maintain tissue homeostasis, and in response to unrepairable damage,
- ‘stick together’ by maintaining selective adhesions that they progressively adjust which ensures they remain in their intended location,
- Differentiate into specialized cells with specific functions meaning they can adopt different physical characteristics despite having the same genome.

Cancer is a complex genetic disease that is caused by specific changes to the genes in one cell or group of cells. These changes disrupt normal cell function – specifically affecting how a cell grows and divides. In contrast to normal cells, cancer cells don't stop growing and dividing, this uncontrolled cell growth results in the formation of a tumour. Cancer cells have more genetic changes compared to

normal cells, however not all changes cause cancer, they may be a result of it. The genetic changes that contribute to cancer usually affect three specific types of gene; proto-oncogenes, tumour suppressor genes, and DNA repair genes.

Normal Cell vs Cancer Cell – The Key Differences

Cell shape: Normal human cells come in many shapes and sizes – as they differentiate and adopt specialized functions their shape changes accordingly – for instance a red blood cell looks very different to a nerve cell. *Different* types of cells do not look alike, but, if you analyse cells of the *same* cell type they will look extremely similar, maintaining a uniform shape.

For years researchers have been peering down microscopes, looking for distinct features that can help them determine the difference between a cancer cell and normal cell. Cancer cells are misshapen, and appear as a chaotic collection of cells, in an array of shapes and sizes. Researchers have been investigating the relationship between cancer cell shape and a patients' outlook, and whether cell shape may also help to distinguish between the different types of cancer.

Nucleus: In normal cells the nucleus has a smooth appearance and maintains a uniform, spheroid shape. Several structural components are involved in the regulation of nuclear morphology. One of these structural components is the nuclear lamina. Cancer cell nuclei are frequently misshapen and bulges known as "blebs" can often be observed in cells' nuclear membranes. Research suggests that this 'blebbing' is caused by an imbalance in the proteins that constitute the nuclear lamina which leads to separation of the lamina fibers.

Chromatin: The fine, evenly distributed chromatin found in normal cells transforms into coarse, chromatin in cancer cells – aggregating into irregular clumps that vary in both size and shape.

Nucleolus: Tumour aggressiveness and clinical outcome can both be measured by observing the morphology of a cancer cell's nucleolus/ nucleoli. The nucleolus becomes increasingly enlarged and more irregular in cancer cells – cells can have multiple nucleoli within the nucleus.

Blood supply: Angiogenesis is defined as the development of new blood vessels that form from pre-existing vasculature. Angiogenesis is a vital process in normal cells that occurs during development, growth, and wound healing. However, it is also implicated in the growth of cancer, through the tumour's ability to secrete chemical signals that stimulate angiogenesis.

	Normal Cell	***Cancer Cell***
Cell shape	Uniform	Irregular
Nucleus	Spheroid shape, single nucleus	Irregular shape, multi-nucleation common
Chromatin	Fine, evenly distributed	Coarse, aggregated
Nucleolus	Single, inconspicuous nucleolus	Multiple, enlarged nucleoli
Cytoplasm	Large cytoplasmic volume	Small cytoplasmic volume
Growth	Controlled	Uncontrolled
Maturation	Mature into specialized cells	Remain immature and undifferentiated
Blood supply	Normal angiogenesis (occurs during development/ healing)	Tumour-induced angiogenesis
Oxygen	Favoured (for aerobic respiration) but will undergo anaerobic respiration if required	Not required (thrive in hypoxic conditions), favour anaerobic respiration
Location	Remain in their intended location	Can spread to different locations in the body (metastasis)

Development of Cancer

The development of cancer is a multistep process in which cells gradually become malignant through a progressive series of alternations. This process involves mutation and selection for cells with progressively increasing capacity for cell division, survival, invasion and metastasis (spread of cancer cells through the blood or lymphatic system to other organ sites).

The first step in the process is when a single cell within a tissue of the organ concerned is genetically modified. The modified cell divides rapidly, although surrounding cells do not— and a mass of tumour cells forms. These cells constitute a clone where cells are identical in terms of structure, characteristics and function. Rapid cell proliferation leads to the tumorous outgrowth or adenoma or polyp. This tumour is still benign.

Tumour progression continues as additional mutation occur within cells of tumour population. Some of these mutations give a selective advantage to the cell such as rapid growth and the descendants of a cell bearing such a mutation will consequently become dominant within the tumour population. This process is known as clonal selection. Clonal selection continues throughout tumour development and, consequently, tumour become more and more rapid, growing and increasingly malignant. The tumour cells, by their rapid proliferation, invades the basal lamina that surrounds the tissue.

Then tumour cells spread into blood vessels that will distribute them to other sites in the body. This is known as metastasis. If the tumour cells can exit from the blood vessels and grow at distant site, they are considered malignant (Fig 1).

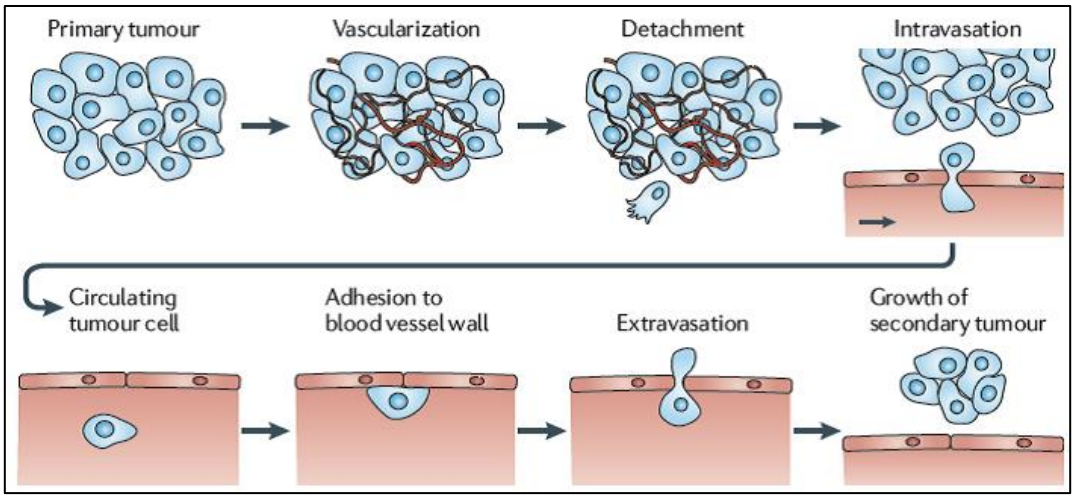

Fig 1: Cancer metastasis

Causes of Cancer

Normal cells can be converted to cancer cells by treatment with a wide variety of chemicals, ionizing radiations and several DNA and RNA-containing viruses.

Broadly speaking, two large groups of viruses appear to be carcinogenic. They include DNA tumour viruses and RNA tumour viruses, depending on the type of nucleic acid present within the mature virus particle. Among DNA tumour viruses, there are simian virus 40 (SV 40), adenovirus, polyoma virus and herpes like virus. The RNA tumour viruses include retroviruses similar to HIV. These are called oncogenic virus.

Two kinds of genes have been found to be associated with carcinogenesis:

These comprise tumour – suppressor genes and oncogenes.

1. Tumour – suppressor genes:

These are anti-oncogenes and encode proteins which restrain cell growth and prevent cells from becoming malignant. First tumour – suppressor gene resulting in eye cancer called retinoblastoma was discovered, designated as RB. It was caused due to deletion in one member of 13th pair of chromosome. Other cancers caused by tumour-suppressor genes include colon carcinoma, nephroblastoma, neurofibromas and thyroid carcinoma etc.

2. Oncogenes:

Oncogenes are genes capable of causing cancer. These were first recognised as unique genes of tumour-causing viruses that are responsible for the process of transformation (viral oncogenes).

There are two classes of oncogenes — one is viral oncogene present in viruses that causes the transformation of target cells. The counterpart of which stays within the host cell involved in normal cellular functions are called cellular oncogenes. The cellular sequences themselves are not oncogenic, are described as proto-oncogenes, whose capture by retrovirus and subsequent modification may create an oncogene.

Oncogenic potential of tumour virus resides in a single function or a group of related functions that are active early in the viral lytic cycle. Tumour viruses carry genes (v-one) which confer on them the ability to convert host cell into tumorigenic state. About 100 viral oncogenes have been identified so far.

When transformation occurs, the relevant genes are integrated into the genomes of transformed cells and expressed constitutively. Oncogenes of DNA tumour viruses do not have cellular counterparts. In case of non- defective RNA viruses, tumourigenicity does not rely upon an individual viral oncogene, but upon the ability of the virus to activate a cellular proto- oncogene.

Acute transforming retroviruses capture cellular oncogene (absent in ancestral virus) by means of a transduction event during an infective cycle. At least 25 c-onc genes have been identified by their representation in retroviruses. Viral infection is not really necessary for tumour formation as evidenced by transfection assay.

Oncogenes of Rous Sarcoma Virus:

(i) The genome of this retrovirus contains four genes named gag, pol, env, and src (Fig 2).

(ii) The gag gene codes for group-specific antigens of the virus, pol for the reverse transcriptase that characterizes retroviruses, and env for certain glycoproteins of the viral envelope. A protein-tyrosine kinase was shown to be the product of src (i.e., the sarcoma-causing gene) that is responsible for transformation.

(iii) Certain glycolytic enzymes become target proteins for the src protein-tyrosine kinase. This shows that transformed cells often show increased rates of glycolysis. The product of src may also catalyze phosphorylation of phosphatidylinositol to phosphatidylinositol mono- and bi-phosphate.

(iv) When phosphatidylinositol 4, 5-bi-phosphate is hydrolyzed by the action of phospholipase C, 2 second messengers are released: inositol triphosphate and diacylglycerol. The first compound mediates release of Ca^{++} from intracellular sites of storage (e.g., the endoplasmic reticulum).

(v) Diacylglycerol stimulates the activity of the plasma membrane-bound proteins kinase C which in turn phosphorylase a number of proteins, some of which may be components of iron pumps.

(vi) Mild alkalinization of the cell brought about by activation of a Na^+/H^+ anti-port system can play a role in stimulating mitosis.

The product of src may, therefore, affect a large number of cellular processes by its ability to phosphorylate various target proteins and enzymes and by stimulating the pathway of synthesis of the polyphosphoinositides.

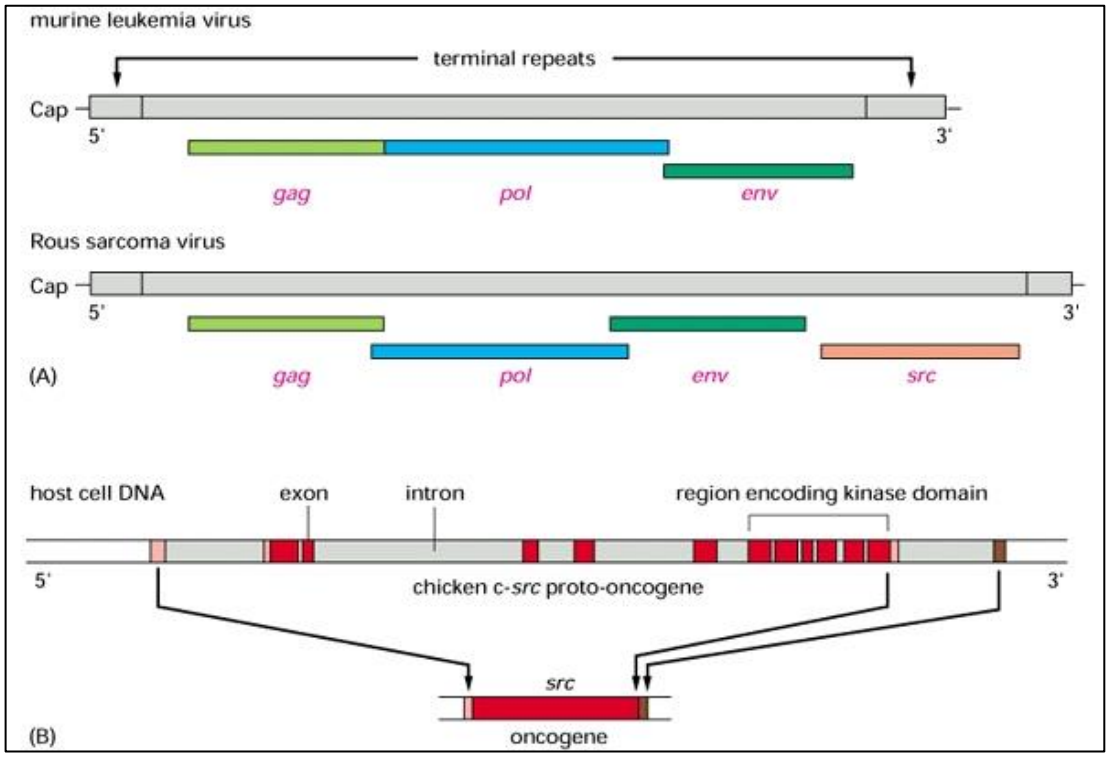

Fig 2: Oncogene of Rous sarcoma virus and its incorporation into host genome

Oncoproteins

Oncoproteins are the product (the proteins) that are coded for by oncogenes and are produced when the gene is transcribed and translated (the process of "writing down the code" on RNA and manufacturing the proteins).

There are many types of oncoproteins depending on the specific oncogene present, but most work to stimulate cell growth and division, inhibit cell death (apoptosis), or inhibit cellular differentiation (the process by which cells become unique). These proteins can also play a role in the progression and aggressiveness of a tumour that is already present.

Types and Examples

Different types of oncogenes have different effects on growth (mechanisms of action), and to understand these it's helpful to look at what is involved in normal cell proliferation (the normal growth and division of cells). Most oncogenes regulate the proliferation of cells, but some inhibit differentiation (the process of cells becoming unique types of cells) or promote survival of cells (inhibit programmed death or apoptosis). Recent research also suggests that proteins produced by some oncogenes work to suppress the immune system, reducing the chance that abnormal cells will be recognized and eliminated by immune cells such as T-cells.

i) Growth Factors

Some cells with oncogenes become self-sufficient by making (synthesizing) the growth factors to which they respond. The increase in growth factors alone doesn't lead to cancer but can cause rapid growth of cells that raises the chance of mutations.

An example includes the proto-oncogene SIS, that when mutated results in the overproduction of platelet-derived growth factor (PDGF). Increased PDGF is present in many cancers, particularly bone cancer (osteosarcoma) and one type of brain tumor.

ii) Growth Factor Receptors

Oncogenes may activate or increase growth factor receptors on the surface of cells (to which growth factors bind).

One example includes the HER2 oncogene that results in a significantly increased number of HER2 proteins on the surface of breast cancer cells. In roughly 25% of breast cancers, HER2 receptors are found in numbers 40 times to 100 times higher than in normal breast cells. Another example is the epidermal growth factor receptor (EGFR), found in around 15% of non-small cell lung cancers.

iii) Signal Transduction Proteins

Other oncogenes affect proteins involved in transmitting signals from the receptor of the cell to the nucleus. Of these oncogenes, the ras family is most common (KRAS, HRAS, and NRAS) found in roughly 20% of cancers overall. BRAF in melanoma is also in this category.

iv) Non-Receptor Protein Kinases

Non-receptor protein kinases are also included in the cascade that carries the signal to grow from the receptor to the nucleus.

A well-known oncogene involved in chronic myelogenous leukemia is the Bcr-Abl gene (the Philadelphia chromosome) caused by a translocation of segments of chromosome 9 and chromosome 22. When the protein produced by this gene, a tyrosine kinase, is continually produced it results in a continuous signal for the cell to grow and divide.

v) Transcription Factors

Transcription factors are proteins that regulate when cells enter, and how they progress through the cell cycle.

An example is the Myc gene that is overly active in cancers such as some leukemias and lymphomas.

vi) Cell Cycle Control Proteins

Cell cycle control proteins are products of oncogenes that can affect the cell cycle in a number of different ways.

Some, such as cyclin D1 and cyclin E1 work to progress through specific stages of the cell cycle, such as the G1/S checkpoint.

vii) Regulators of Apoptosis

Oncogenes may also produce oncoproteins that reduce apoptosis (programmed cell death) and lead to prolonged survival of the cells.

An example is Bcl-2, an oncogene that produces a protein associated with the cell membrane that prevents cell death (apoptosis)

How Cellular Oncogenes Arise

Oncogenes can arise inside cells in two fundamentally different ways.

(i) One mechanism involves the participation of cancer viruses that introduce oncogenes into the cells they infect.

(ii) The alternative, is based on a series of mechanisms that convert normal cellular genes into oncogenes, often as a result of exposure to carcinogenic agents.

Cellular Oncogenes Arise from Proto-Oncogenes

Those normal cellular genes that can be converted into oncogenes are referred to as proto-oncogenes. Despite their harmful-sounding name, proto-oncogenes are not bad genes simply lying in wait for an opportunity to foster the development of cancer. Rather they are normal genes that make essential contributions to the regulation of cell proliferation and survival.

The term proto-oncogene simply implies that if and when the structure or activity of a proto-oncogene is disrupted by certain kinds of mutations, the altered form of the gene can cause cancer. In genetic terms, such mutations are considered to be **"gain-of-function"** mutations because they create a new function, namely the ability to induce tumour formation that is not originally present in proto-oncogenes. Thus proto-oncogenes are normal genes that contribute to normal cell function, but they can also be converted into oncogenes, which are dysfunctional genes that produce proteins that perform a decidedly abnormal function—that is, inducing the development of cancer.

Proto-oncogenes

Proto-oncogenes are normal genes that help cells grow. A proto-oncogene can't cause cancer unless a mutation occurs in the gene that turns it into an oncogene.

When a mutation occurs in a proto-oncogene, it becomes permanently turned on (activated). The gene will then start to make too much of the proteins that code for cell growth. Cell growth occurs uncontrollably. This is one of the defining features of cancerous tumors.

Everyone has proto-oncogenes in their body. In fact, proto-oncogenes are necessary for our survival. Proto-oncogenes only cause cancer when a mutation occurs in the gene that results in the gene being permanently turned on. This is called a gain-of-function mutation.

These mutations are also considered dominant mutations. This means that only one copy of the gene needs to be mutated in order to encourage cancer.

There are at least three different types of gain-of-function mutations that can cause a proto-oncogene to become an oncogene:

- **Point mutation.** This mutation alters, inserts, or deletes only one or a few nucleotides in a gene sequence, in effect activating the proto-oncogene.
- **Gene amplification.** This mutation leads to extra copies of the gene.
- **Chromosomal translocation.** This is when the gene is relocated to a new chromosomal site that leads to higher expression.

Some of these mutations result from an infection with a type of virus called a retrovirus. Radiation, smoke, and other environmental toxins may also play a role in causing mutation in proto-oncogenes. As well, some people are more susceptible to mutations in their proto-oncogenes.

Examples of proto-oncogenes

Over 40 different proto-oncogenes have been discovered in the human body. Examples include:

Ras: The first proto-oncogene to be shown to turn into an oncogene is called *Ras*. *Ras* encodes an intracellular signal-transduction protein. In other words, *Ras* is one of the on/off switches in a series of steps in a major pathway that eventually leads to cell growth. When *Ras* is mutated, it encodes for a protein that causes an uncontrolled growth-promoting signal.

Most cases of pancreatic cancer have a point mutation in the *Ras* gene. Many cases of lung, colon, and thyroid tumours have also been found to have a mutation in *Ras*.

HER2: Another well-known proto-oncogene is *HER2*. This gene makes protein receptors that are involved in the growth and division of cells in the breast. Many people with breast cancer have a gene amplification mutation in their *HER2* gene. This type of breast cancer is often referred to as *HER2*-positive breast cancer.

Myc: The *Myc* gene is associated with a type of cancer called Burkitt's lymphoma. It occurs when a chromosomal translocation moves a gene enhancer sequence near the *Myc* proto-oncogene.

Cyclin D: *Cyclin D* is another proto-oncogene. Its normal job is to make a protein called Rb tumor suppressor protein inactive. In some cancers, like tumours of the parathyroid gland, *Cyclin D* is activated due to a mutation. As a result, it can no longer do its job of making the tumor suppressor protein inactive. This in turn causes uncontrolled cell growth.

Oncogene in Human Cancer

Direct evidence for the involvement of cellular oncogenes (the term cellular oncogene is generally used to distinguish this group of cancer- causing genes from viral oncogenes) in human tumour was first derived from gene transfer experiment carried out in the laboratories of Robert Weinberg and Geoffrey Cooper in the early 1980s.

In this process, a DNA segment isolated from tumour cells are artificially introduced into normal cells to see its subsequent changes. DNA isolated from a human bladder carcinoma was found to efficiently induce malignant transformation of recipient mouse cells in culture. This experiment reveals that the human tumour contains a cellular oncogene.

The first human oncogene identified in gene transfer experiment was the ras oncogene. The ras oncogenes are not present in normal cells, but they are generated in tumour cells as a consequence of point mutation of the ras proto-oncogene. This results in the change of a single amino acid at critical position of the ras protein molecule encoded by ras gene.

The first such mutation was the substitution of valine for glycine at position 12. A single nucleotide, change which alters codon 12 from GGC (Gly) to GTC (Val) is responsible for the transforming activity. This is detected in bladder carcinoma DNA. The ras gene encodes membrane-bound guanine-nucleotide binding proteins (G- protein) that plays a central role in the transmission of singles from receptor-bound external growth factor to the cell interior. During this process, GTP is hydrolysed into GDP. Therefore, Ras protein alternates between active (GTP bound) and inactive (GDP bound) states. But oncogenic ras proteins remain in the active GTP bound state and drive unregulated cell proliferation leading to the development of malignancy.

In human tumour, point mutation is an important mechanism by which proto-oncogenes are converted into oncogenes. Besides this, the gene rearrangement—resulting mainly from chromosome translocation—sometimes lead to the conversion of proto-oncogene to oncogene.

The classical example regarding the conversion of proto-oncogene to oncogene due to translocation of chromosome is the **Burkitt's lymphoma**. It produces the malignancy of the antibody producing B-lymphocytes.

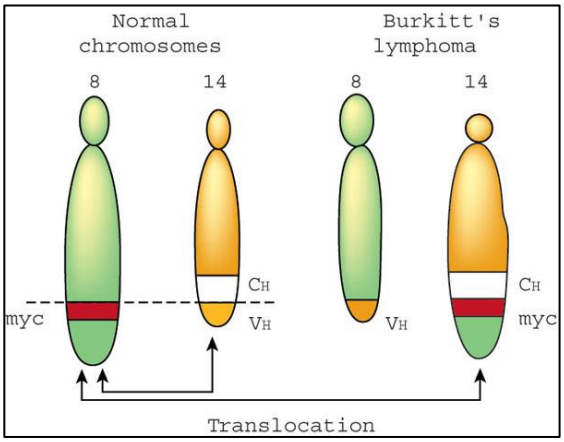

Fig 3: Burkitt's lymphoma

In this case a piece of chromosome(s) 8 carrying c-myc proto-oncogene is trans-located to the immunoglobulin heavy chain locus on chromosome 14 (Fig 3). Since the antibody genes are extremely active in lymphocytes, the transcriptional regulation of the adjacent myc proto-oncogene is disturbed, resulting in an abnormal pattern of synthesis of the myc protein product.

Such abnormal pattern of expression of the c-myc gene—which encodes transcription factor normally induced in response to growth factor stimulation—is sufficient to drive cell proliferation and contribute to tumour development.

Translocation of some proto-oncogene often causes the rearrangement of coding sequences which lead to the formation of abnormal gene products. In chronic myelegenous leukemia, the abl proto-oncogene is trans-located from chromosome 9 to chromosome 22 forming **Philadelphia chromosome** (Fig 4).

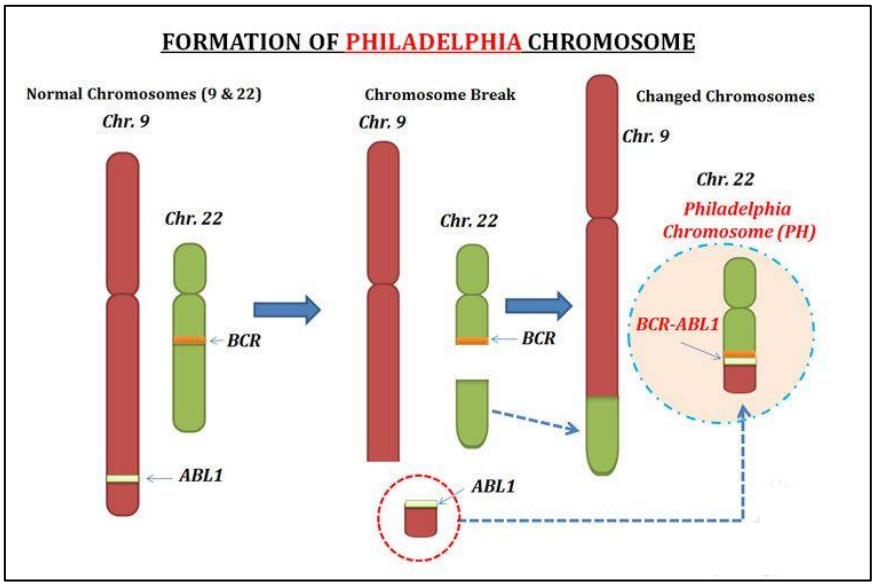

Fig 4: Philadelphia chromosome

The abl proto- oncogene which contains two alternative first exon (1A and IB) is joined to the middle to the bcr gene on chromosome 22. Exon IB is deleted as a result of the translocation. Transcription of

the fused gene initiates at the bcr promotor and continues through abl. Splicing then generates a fused bcr/abl mRNA, in which abl exon 1A sequences are also deleted and bcr sequences are joined to abl Exon 2.

The bcr/abl mRNA is translated to yield a recombinant bcr/abl fusion protein in which the normal amino terminus of abl proto-oncogene has been replaced by bcr amino acid sequences. The fusion of bcr sequences results in aberrant activity and altered subcellular localisation of the abl protein tyrosine kinase, leading to cell transformation.

Gene amplification occurring in the tumour cell is a common process by which proto- oncogenes are converted to oncogene. Gene amplification takes place due to an increase of the number of copies of a gene resulting from the repeated replication of a region of DNA. Therefore, gene amplification leads to the overproduction of a particular protein or enzyme from the amplified gene. A prominent example of oncogene amplification is the involvement of the N-myc gene in neuroblastoma, a tumour of embryonal neuronal cells.

Probable questions:

1. Write down the differences between benign and malignant tumour.
2. Write down the characteristics of cancer cells.
3. What is lymphoma?
4. Write short notes on 'loss of contact inhibition'.
5. Describe the oncogene of Rous sarcoma virus and its incorporation into host genome.
6. What is protooncogene?
7. What do you mean by Burkitt's lymphoma?
8. What is oncoproteins? Give eamples

Suggested readings:

1. Hardin, J. Bertoni, G and Klein smith, J. L. (2012). Becker's World of the Cell. 8th Edn, Pearson Benjamin Cummings, San Francisco.

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3. Plopper, G, D. Sharp, Siroski, E (2015) Lewin's Cell 3rdEdition—Johns & Bartlett Publishers

4. Pollard and Earnshaw (2007). Cell Biology. 2nd. Edn Saunders.

5. Albert Bruce, Bray Dennis, Levis Julian ,Raff Martin, Roberts Keith and Watson James (2008).Molecular Biology of the Cell, V Edition, Garland publishing Inc., New York and London.

6. Cooper, G.M. and Hausman, R.E. (2009). The Cell: AMolecularApproach.5thEdition. ASM Press and Sunderland, Washington, D.C.; Sinauer Associates, MA.

Unit IV

Classification and characteristics of chemical carcinogen; role of radiation and DNA repair in carcinogenesis.

Objective: In this unit you will know about classification and characteristics of chemical carcinogen; role of radiation and DNA repair in carcinogenesis.

Introduction

Many agents including radiation, chemicals and viruses have been found to induce cancer in both experimental animals and humans. Agents which cause cancers are called carcinogens. They can be divided into three major categories: chemical carcinogens (including those from biological sources), physical carcinogens, and oncogenic (cancer-causing) viruses.

Chemical carcinogens act by damaging DNA and inducing somatic mutations. These carcinogens are generally called initiating agent because the induction of mutations in key target genes is supposed to be the initial event leading to cancer development. Some of the initiating agents that cause human cancers include solar ultraviolet radiation—the major cause of skin cancer. The exposure of the thyroid gland to X-rays greatly increases the incidence of thyroid cancers.

Varieties of chemical carcinogen including tobacco smoke (containing benzo(a)pyrene, dimethyl nitrosamine and nickel compound) and aflatoxin produced by some molds are the major identified cause of human cancer. Other carcinogens induce the cancer development by stimulating cell proliferation rather than inducing mutations. Such compounds axe called tumour promoters.

Earlier study

The first suggestion that chemicals can cause cancer dates back to 1761, when a doctor noted that people who use snuff suffer from nasal cancer. A few years later a British physician observed a high incidence of cancer of the scrotum among the chimney-sweepers iii their youth. He explained the fact that the chimney soot became dissolved in the natural oil of the scrotum, irritating the skin and, consequently, initiates the development of cancer. On the basis of two separate observations it became evident that certain chemicals can cause cancer.

In the early 1940s Peyton Rous observed that repeated application of coal tar to rabbit skin causes tumour to develop, but the tumour disappears when application of the coal tar is stopped. It is also noted that when the skin is treated with turpentine, tumour again reappears. Normally turpentine does not cause cancer itself. Therefore the coal tar and turpentine are playing two different roles. Some carcinogens induce some normal cells to become irreversibly altered to a pre-neoplastic state.

This is known as initiation and the carcinogens are known as initiation agents. Here coal tar is an initiating agent. On the other hand, some carcinogens stimulate the pre-neoplastic cells to divide and form tumour. This is known as promotion and the carcinogens are termed promoting agents. Here turpentine behaves as promoting agents.

Identifying Chemicals that Cause Cancer

We seem to be surrounded by a sea of chemical carcinogens. They are found in the air we breathe, the food we eat, the water and beverages we drink, the medications we take, the places where we work, and the homes in which we live. However, this assessment—while technically correct—conveys the

misimpression that we are faced with severe hazards everywhere we look and that these dangers cannot be avoided. In fact, many of the carcinogens we normally encounter are only weakly carcinogenic, and most of the more potent ones can be easily avoided by the general public. So rather than lumping all chemical carcinogens together, we need to consider them as individual molecules and make informed judgments about the dangers posed by each one.

Classification of chemical carcinogen

The carcinogenic chemicals that act as initiating agent are capable to bind with DNA. Hence they interfere with the normal function of DNA and induce somatic mutation and, consequently, bring about stable, inheritable changes in the cell's properties.

I. On the basis of action of chemical carcinogens on DNA, there are two broad categories of carcinogens—direct acting and indirect acting (Fig 1).

Direct acting carcinogens are highly electrophilic compounds that react with DNA. Indirect acting carcinogens are converted to ultimate carcinogens by introduction of electrophilic centres. In other words, indirect acting carcinogens must be metabolised before they can react with DNA.

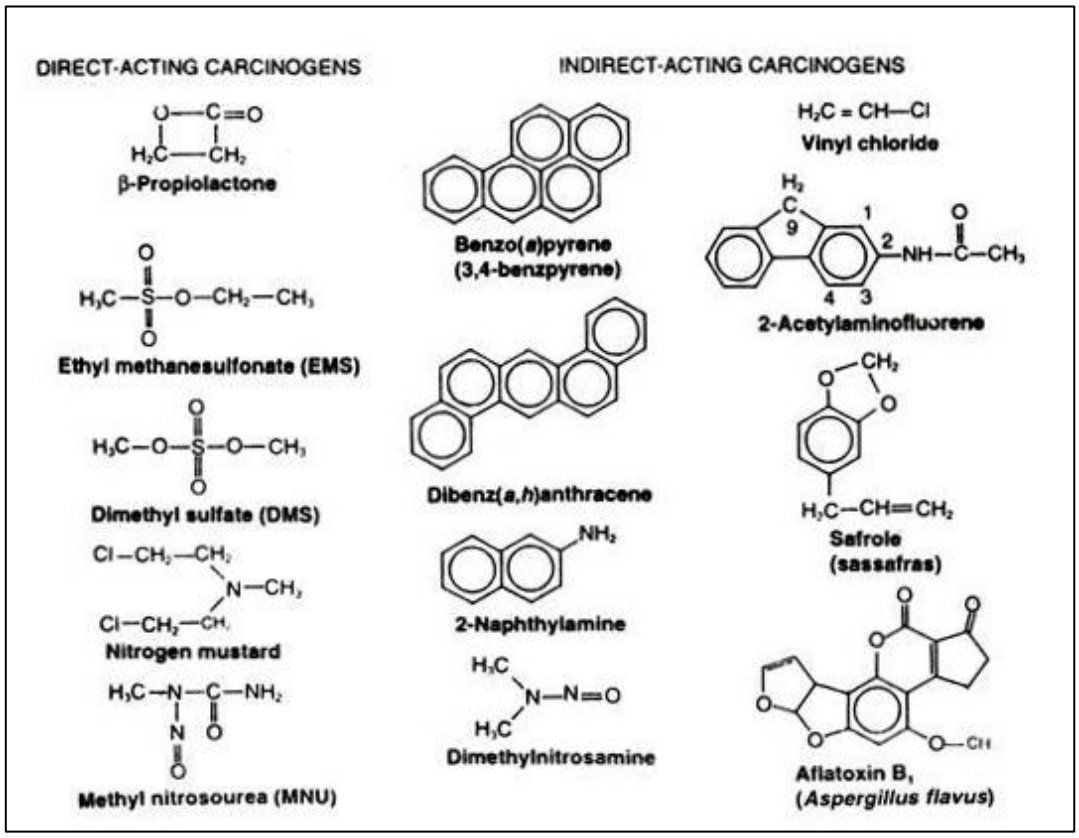


Fig 1: Structure of some direct acting and indirect acting chemical carcinogens

II. The vast majority fall into one of the following five categories depending in the carbon-containing compounds. They range from small organic molecules containing only a few carbon atoms to large, complex molecules constructed from multiple carbon-containing rings (Fig 2).

1. Carcinogenic polycyclic aromatic hydrocarbons (or simply polycyclic hydrocarbons) are a diverse group of compounds constructed from multiple, fused benzene rings. Polycyclic hydrocarbons are

natural components of coal tars, soots, and oils, and are also produced during the incomplete combustion of coal, oil, tobacco, meat, and just about any other organic material that can be burned.

The carcinogenic potency of polycyclic hydrocarbons varies widely, from weak or noncarcinogenic molecules to very potent carcinogens. The polycyclic hydrocarbons benzopyrene and dibenzoanthracene, isolated from coal tar in the 1930s, were the first purified chemical carcinogens of any kind to be identified.

2. Carcinogenic aromatic amines are organic molecules that possess an amino group (—NH_2) attached to a carbon backbone containing one or more benzene rings. Some aromatic amines are aminoazo compounds, which means that they contain an azo group (N=N) as well as an amino group. Among the carcinogens in these categories are the aromatic amines benzidine, 2-naphthylamine, 2-acetylaminofluorene, and 4-aminobiphenyl, and the aminoazo dyes 4- dimethylaminoazobenzene and o-aminoazotoluene.

Many of these compounds were once employed in the manufacturing of dyes, although most are no longer used in significant quantities because of their toxicity. Some aromatic amines, such as 2-naphthylamine and 4-aminobiphenyl, are components of tobacco smoke. As in the case of polycyclic hydrocarbons, the carcinogenic potency of aromatic amines and aminoazo dyes varies from substances that are strongly carcinogenic to substances that are not carcinogenic at all.

3. Carcinogenic N-nitroso compounds are organic chemicals that contain a nitroso group (N=O) joined to a nitrogen atom. Members of this group include the nitrosamines and nitrosoureas, which are potent carcinogens when tested in animals. Most of these compounds are industrial or research chemicals encountered mainly in the workplace, although a few are present in cigarette smoke.

Nitrates and nitrites used in the curing of meats, which are not carcinogenic in themselves, can be converted in the stomach into nitrosamines, but no consistent relationship between these compounds and human cancer has been established.

4. Carcinogenic alkylating agents are molecules that readily undergo reactions in which they attach a carbon-containing chemical group to some other molecule. Unlike the three preceding groups of carcinogens, which are defined by their chemical structures (i.e., the presence of multiple benzene rings, amino groups, or nitroso groups), alkylating agents are defined not by their structural features but by their chemical reactivity—that is, their ability to join a chemical group to another molecule. The N-nitroso compounds, discussed in the preceding paragraph, are examples of carcinogens that function as alkylating agents.

Other examples include vinyl chloride (used in the production of plastics) and ethylene oxide (used in the production of antifreeze and other chemicals). Vinyl chloride and ethylene oxide are among the highest-volume chemicals produced in the United States. Other carcinogenic alkylating agents include sulfur mustard (a chemical warfare agent) and several drugs used in cancer chemotherapy.

5. Carcinogenic natural products are a structurally diverse group of cancer-causing molecules produced by biological organisms, mainly microorganisms and plants. Included in this category is aflatoxin, a carcinogenic chemical made by the mold Aspergillus. One of the most potent carcinogens known, aflatoxin sometimes contaminates grains and nuts that have been stored under humid conditions. Other carcinogenic natural products include plant-derived molecules such as safrole, a major component of sassafras root bark, and pyrrolizidine alkaloids, produced by a variety of different plants.

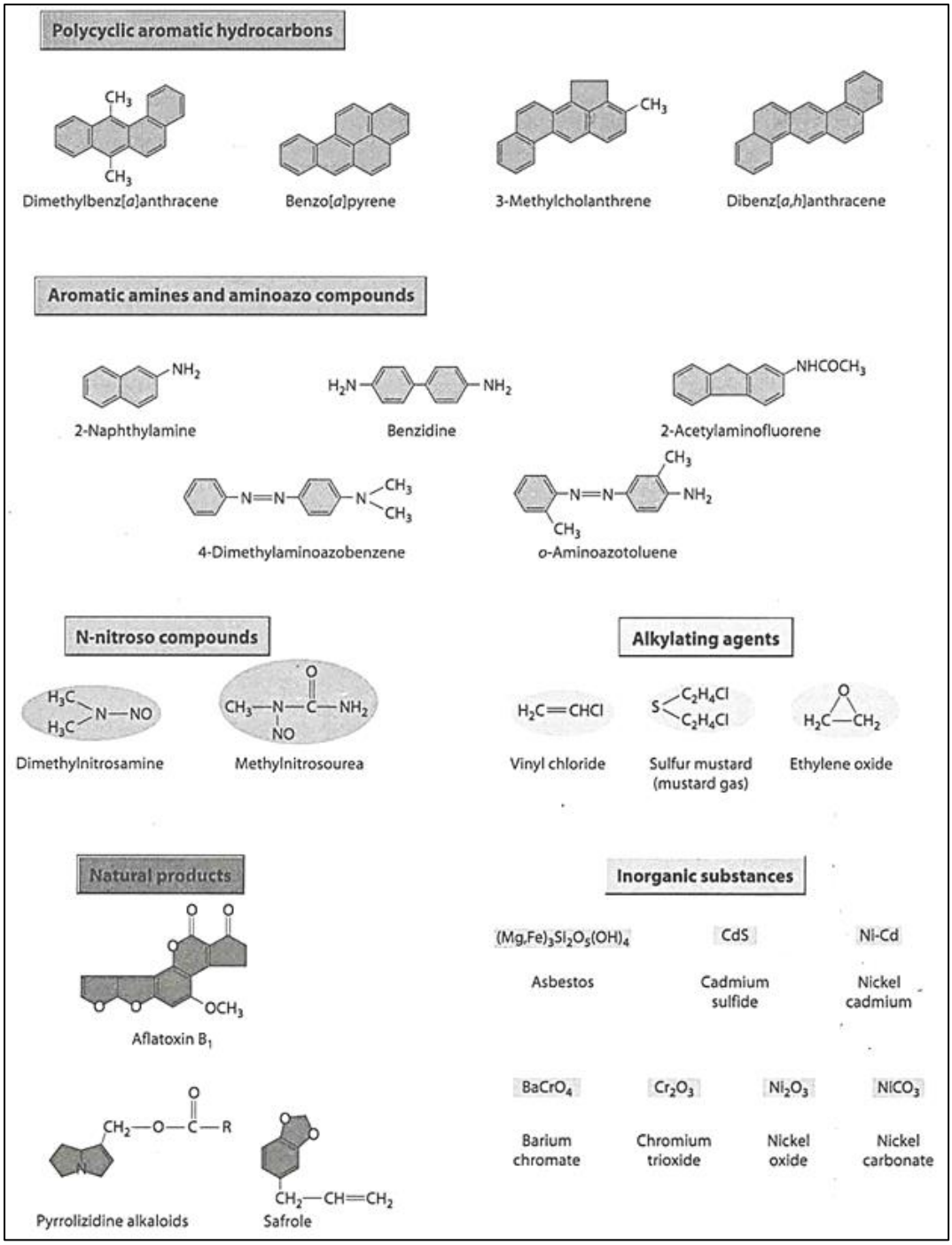


Fig 2: Main classes of carcinogenic chemicals

In addition to the preceding five classes of organic molecules, a small number of *inorganic substances (compounds without carbon and hydrogen) are carcinogenic*. Included in this group are compounds containing the metals cadmium, chromium, and nickel.

Some inorganic substances appear to be carcinogenic in the absence of chemical reactivity. For example, **asbestos** is a mineral composed of silicon, oxygen, magnesium, and iron, but its ability to cause cancer is related to the crystal structure and size of the microscopic fibers it forms rather than their precise chemical makeup.

Characteristics of chemical carcinogens

	Characteristic	Examples of relevant evidence
1.	**Is electrophilic or can be metabolically activated**	Parent compound or metabolite with an electrophilic structure (e.g., epoxide, quinone), formation of DNA and protein adducts
2.	**Is genotoxic**	DNA damage (DNA strand breaks, DNA–protein cross-links, unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (e.g., chromosome aberrations, micronuclei)
3.	**Alters DNA repair or causes genomic instability**	Alterations of DNA replication or repair (e.g., topoisomerase II, base-excision or double-strand break repair)
4.	**Induces epigenetic alterations**	DNA methylation, histone modification, microRNA expression
5.	**Induces oxidative stress**	Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g., DNA, lipids)
6.	**Induces chronic inflammation**	Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production
7.	**Is immunosuppressive**	Decreased immunosurveillance, immune system dysfunction
8.	**Modulates receptor-mediated effects**	Receptor in/activation (e.g., ER, PPAR, AhR) or modulation of endogenous ligands (including hormones)
9.	**Causes immortalization**	Inhibition of senescence, cell transformation
10.	**Alters cell proliferation, cell death or nutrient supply**	Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis
Abbreviations: AhR, aryl hydrocarbon receptor; ER, estrogen receptor; PPAR, peroxisome proliferator–activated receptor. Any of the 10 characteristics in this table could interact with any other (e.g., oxidative stress, DNA damage, and chronic inflammation), which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone.		

Role of radiation and DNA repair in carcinogenesis

Carcinogenesis, also called oncogenesis or tumorigenesis, is the formation of a cancer, whereby normal cells are transformed into cancer cells. The process is characterized by changes at the cellular, genetic, and epigenetic levels and abnormal cell division.

Radiation carcinogenesis is a biological phenomenon whereby living normal cells are damaged by ionizing radiations, which starts a progressive process causing the surviving cells to change their phenotype such that normal controls of cell death and apoptosis are lost and uncontrolled cancerous growth is initiated.

Several types of radiation triggers the development of cancer. Like carcinogenic chemicals, radiation is another source of cancer risk routinely encountered in the environment. Radiation is defined simply

as energy traveling through space. There are many different types of radiation, each defined by its wavelength and energy content. Natural sources of radiation include ultraviolet radiation from the sun, cosmic rays from outer space, and emissions from naturally occurring radioactive elements.

Medical, industrial, and military activities have created additional sources of radiation, mainly in the form of X-rays and radioactivity. Among the various types of radiation, two main classes have been clearly identified as causes of cancer- ultraviolet radiation and ionizing radiation.

The ability of **ultraviolet radiation** to cause cancer was first deduced from the observation that skin cancer is most prevalent in people who spend long hours in the sun and is more frequent in geographical areas where the sunlight is especially intense. Because ultraviolet radiation is absorbed by normal skin pigments, dark-skinned individuals have lower rates of skin cancer than do fair-skinned individuals.

Exposure to sunlight rarely causes any type of malignancy other than skin cancer because ultraviolet radiation is too weak to pass through the skin and into the interior of the body. Fortunately, the most common types of skin cancer rarely metastasize, and their superficial location makes these cancers relatively easy to remove surgically.

During the 1780s, the British House of Commons decided to deal with overcrowding in British jails by banishing criminals to the (then) remote island of Australia. Within a few decades, the east coast of Australia came to be inhabited by light-skinned British men and women whose descendants now represent a large part of the Australian population (Fig 3). The white skin and fair complexion of these people makes them particularly vulnerable to the intense Australian sunlight, and as a result, the white population of Australia has the highest skin cancer rate of any people in the world. Such high rates cannot be explained by hereditary factors because in England, where the sun is weaker and often covered by clouds, this same group of people had low skin cancer rates.

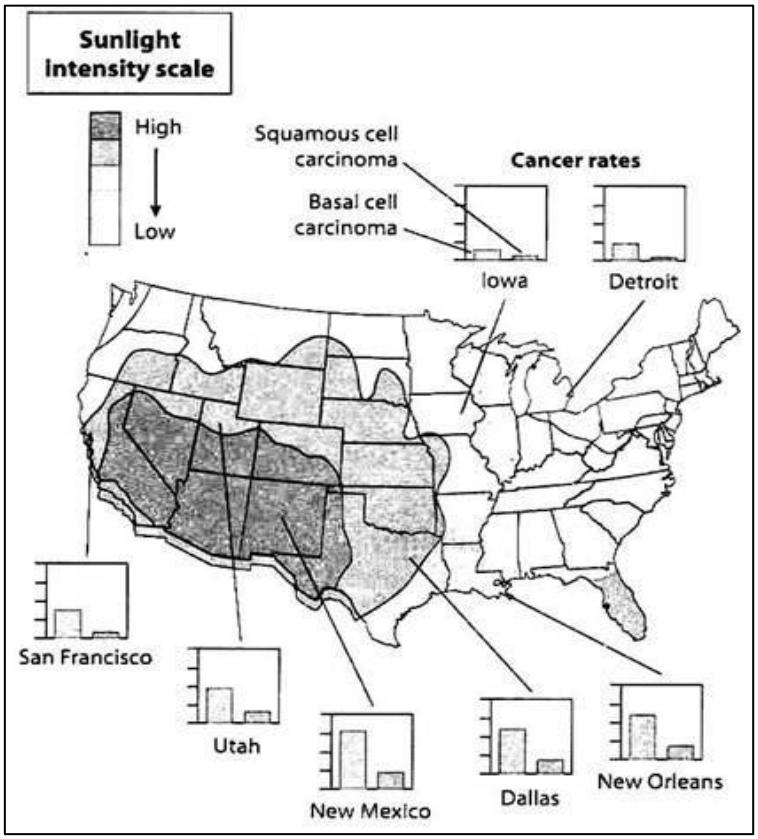


Fig 3: Relationship between skin cancer and sunlight exposure

Ionizing radiation poses a more serious cancer hazard because it is strong enough to penetrate through the skin and reach internal organs. The first type of ionizing radiation found to be a cancer hazard was X-rays, which were discovered in 1895 by Wilhelm Roentgen. Shortly thereafter, people working with X-rays began to develop cancer in unexpectedly high numbers. Another form of ionizing radiation, called **nuclear radiation**, is emitted by radioactive elements.

Sunlight Contains Several Classes of UV Radiation:

To explain how sunlight causes cancer, we need to describe the types of radiation given off by the sun. The sunlight that reaches the earth contains several forms of electromagnetic radiation, which is defined as waves of electric and magnetic fields that are propagated through space at the speed of light. Electromagnetic radiation occurs in a variety of forms that differ in wavelength and energy content (Fig 4).

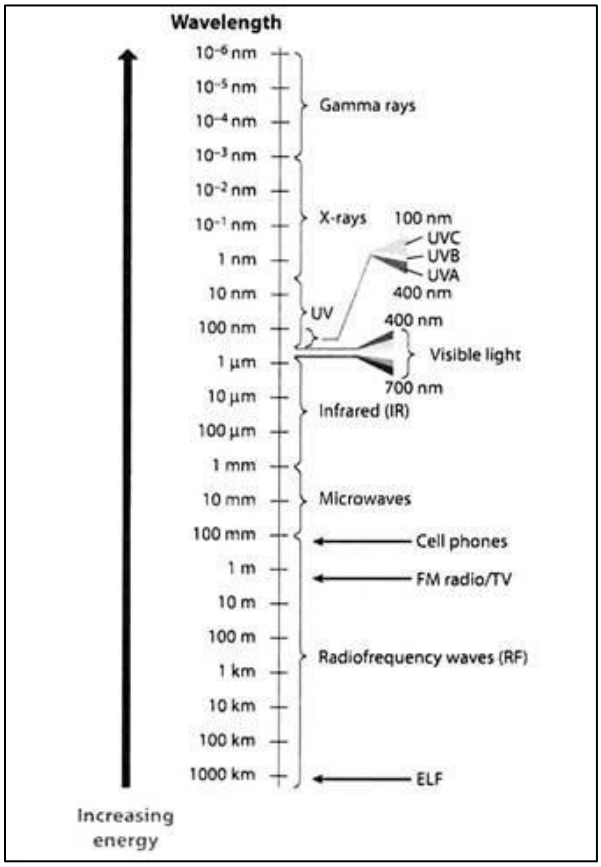


Fig 4: Electromagnetic spectrum

Wavelength and energy are inversely related to each other—that is, radiation of shorter wavelength possesses more energy than radiation of longer wavelength. The longest-wavelength component of sunlight is infrared radiation, which creates the warmth we feel from the sun.

Wavelength and energy are inversely related to each other—that is, radiation of shorter wavelength possesses more energy than radiation of longer wavelength. The longest-wavelength component of sunlight is infrared radiation, which creates the warmth we feel from the sun.

Next comes *visible light*, which is of shorter wavelength than infrared radiation and provides the illumination that allows us to see colors. Finally, ultraviolet radiation (UV) is the shortest-wavelength component of sunlight and possesses the greatest energy, making it capable of inflicting damage on human tissues. The *ultraviolet radiation* in sunlight is in turn subdivided into three classes—A, B, and

C—in order of decreasing wavelength (Table 1). UVA has the longest wavelength and the least energy. Defined as the portion of the UV spectrum whose wavelength falls between 315 and 400 nanometers (nm), UVA is the predominant type of ultraviolet radiation to reach the earth because it is not filtered out by the earth's atmosphere.

Table 1 Types of Ultraviolet Radiation in Sunlight

Type	Wavelength	Properties
UVA	315–400 nm	• Not filtered by ozone layer • Causes skin aging • Stimulates cell proliferation
UVB	280–315 nm	• Partially filtered by ozone layer • Causes sunburn, tanning, skin cancer
UVC	100–280 nm	• Filtered out by ozone layer • Artificial sources cause skin burns and skin cancer

UVA was once thought to be harmless because of its lower energy content, but long-term exposure to UVA is now known to cause aging of the skin and to act as a promoting agent for skin cancer by stimulating cell proliferation.

UVB radiation is of higher energy than UVA, falling in the wavelength range of 280 to 315 nm. Animal studies have shown the UVB is largely responsible for the carcinogenic properties of sunlight. More than 90% of the UVB radiation emitted by the sun is absorbed by ozone molecules present in the upper atmosphere, but enough UVB passes through to the earth's surface to cause sunburn, tanning, aging of the skin, and skin cancer.

Finally, UVC falls in the wavelength range of 100 to 280 nm and is the most energetic type of UV radiation emitted by the sun. This high-energy, short-wavelength form of UV radiation can cause severe burns, but it is completely absorbed by the upper layers of the atmos-phere before reaching the earth. UVC radiation is generally encountered only from artificial light sources, such as the germicidal lamps that use UVC to destroy bac-teria when sterilizing medical and scientific equipment.

UVB Radiation Creates Pyrimidine Dimers in DNA

UVB is the highest-energy component of sunlight to reach the earth, but its energy level is still relatively low and thus it cannot penetrate very far into the body. Instead, UVB is absorbed by cells located in the outer layers of the skin, which explains why sunlight rarely causes any type of malignancy other than skin cancer. The damaging effects of UVB on skin cells often precede the development of cancer by many years.

For example, consider what happens to people who move from England, with its weak sunlight and cloudy skies, to the intensely sunny climate of Australia. Those who move to Australia when they are young develop skin cancer at high rates when they reach middle age, whereas those who move to Australia later in life retain the low skin cancer rates that are typical of people who remain in England. Such observations suggest that skin cancers observed later in life are the result of sunlight damage that occurred many years earlier.

Such a pattern is reminiscent of the initiation phase of chemical carcinogenesis, in which carcinogens trigger DNA mutations that persist for many years, passed from one cell generation to the next as genetically damaged cells proliferate and give rise to tumours. By analogy, researchers have looked to see whether sunlight causes skin cell mutations early in life that can be linked to the later development of cancer.

This is a complicated task because even if mutations are discovered in skin cancer cells, how can you be certain that sunlight caused them? A useful clue has come from studying the interactions of UVB—the main carcinogenic component of sunlight— with different kinds of cells and viruses. The shorter wavelengths of UVB (near 280 nm) are absorbed by the DNA bases, imparting enough energy to alter chemical bonds.

The most common reaction occurs in regions containing the bases cytosine (C) and thymine (T), a class of single-ring bases known as pyrimidines. In loca-tions where two of these pyrimidine bases lie next to each other, absorption of UVB radiation triggers the formation of covalent bonds between the adjacent bases, creating a unique type of mutation called a pyrimidine dimer. All four combinations of two adja-cent pyrimidines—that is, CC, CT, TC, and TT—are frequently converted into covalently linked dimers by UVB radiation.

Although cells can repair pyrimidine dimers, repair needs to occur before DNA replication creates a perma-nent, non-correctable mutation. Figure 4 illustrates how such a permanent mutation could arise, using a CC dimer as an example. During DNA replication, the DNA strand in the region of the CC dimer is distorted and therefore tends to pair improperly with bases in the newly forming DNA strand.

Instead of pairing correctly with its comple-mentary base G, the base C in a CC dimer often pairs incorrectly with the base A (Fig 5 step②). During the next round of DNA replication, the incorrectly inserted A will then form a base pair with its normal complementary base, T, creating an AT base pair Figure 4, step ③).This AT base pair now looks normal to the cellular DNA repair machinery and so will continue to be replicated as if no error had been introduced.

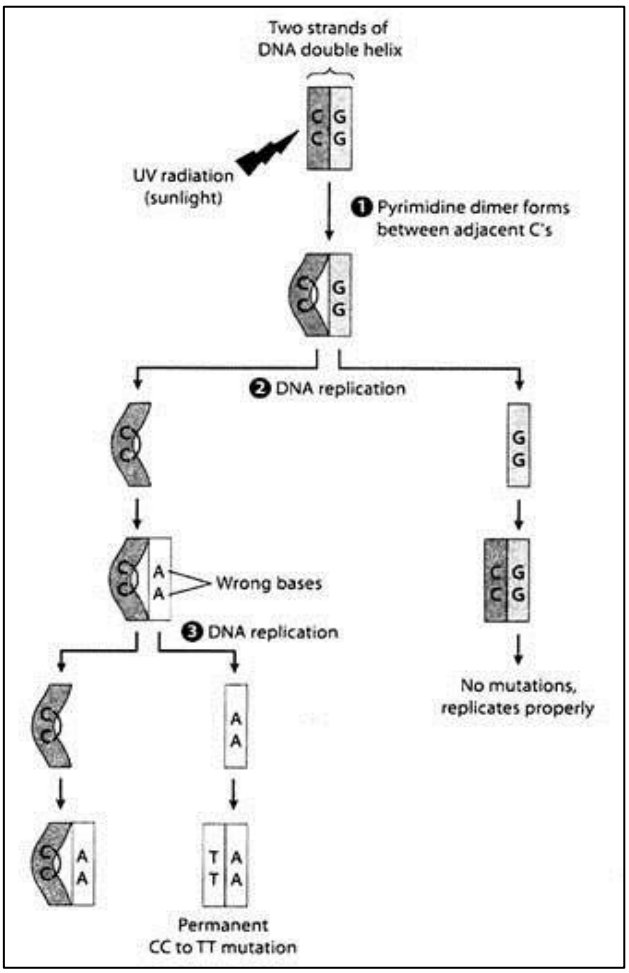


Fig 5: Conversion of a Pyrimidine Dimer into a permanent mutation

Because the base T resides where the base C had been located in the original DNA molecule, the preceding type of mutation is called a C→T substitution. In some cases both C's of the dimer are replaced by the same mecha-nism, creating a CC→TT mutation.

At this point the initial CC dimer in the original DNA strand could be repaired, but the C→T or CC →TT substitution in the newly replicated DNA will be permanent. Such base sub-stitution patterns involving adjacent pyrimidines are unique to UV radiation and are therefore used as a dis-tinctive "signature" to identify mutations caused by sunlight.

Mutations in the p^{53} Gene Triggered by UVB Radiation can lead to Skin Cancer

After scientists discovered that UV radiation selectively induces the formation of pyrimidine dimers, the next task was to determine whether these mutations are associated with skin cancer. Among the first genes to be examined for the presence of pyrimidine dimers was the p^{53} gene, a gene chosen for study because it is known to be mutated in many kinds of human cancer.

When skin cancer cells are examined for the presence of p^{53} mutations, nonmelanoma skin cancers are routinely found to exhibit p^{53} mutations with the distinctive UV **"signature"**—that is, C →T or CC → TT substitutions at dipyrimidine sites. In contrast, the p^{53} mutations arising in cancers of internal body organs do not generally exhibit this UV-specific pattern (Figure 6).

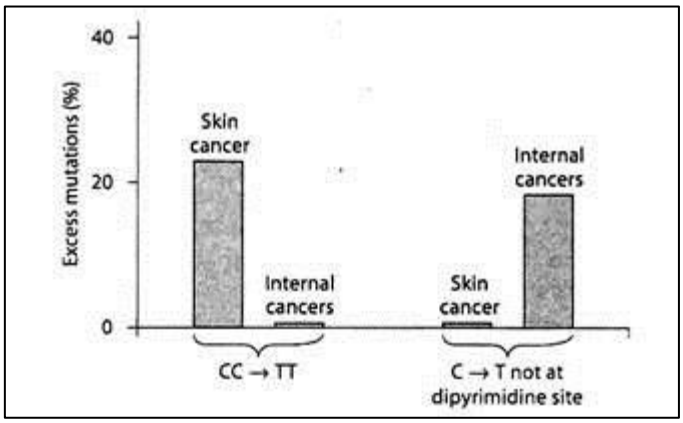


Fig 6: Incidence of two types if p^{53} mutations in skin cancer and internal cancer

The preceding observations indicate that the p^{53} mutations seen in nonmelanoma skin cancer cells are triggered by sunlight, but do these mutations actually cause cancer to arise, or are they simply an irrelevant by-product of long-term exposure to sunlight? This question can be resolved by looking at the precise location of UV-induced mutations within the p^{53} gene. The DNA base sequence of most genes is arranged in a series of three-base units called codons, each of which specifies a particular amino acid in the protein encoded by the gene. Typically, the first two bases of a codon are more important in determining the amino acid than is the third. For example, the codons GAA and GAG both specify the same amino acid (glutamine), so changing the third base from A to G in this codon does not change the amino acid. A similar principle applies to the codons for many other amino acids.

If the p^{53} mutations seen in non-melanoma skin cancers were simply a random by-product of sunlight exposure, mutations in a codons third base (which do not change an amino acid) should be as frequent as mutations in the first or second base (which do change an amino acid). In fact, DNA sequencing has revealed that p^{53} mutations are not randomly distributed but instead involve base changes that alter amino acids.

In other words, the p^{53} mutations seen in non-melanoma skin cancers alter the amino acid sequence of the protein encoded by the p^{53} gene, as would be expected if these mutations are involved in the

mechanism by which sunlight causes cancer. The p^{53} gene is not, however, the only mutant gene to be involved in non-melanoma skin cancers, nor is it as frequently mutated in melanomas (Fig 7).

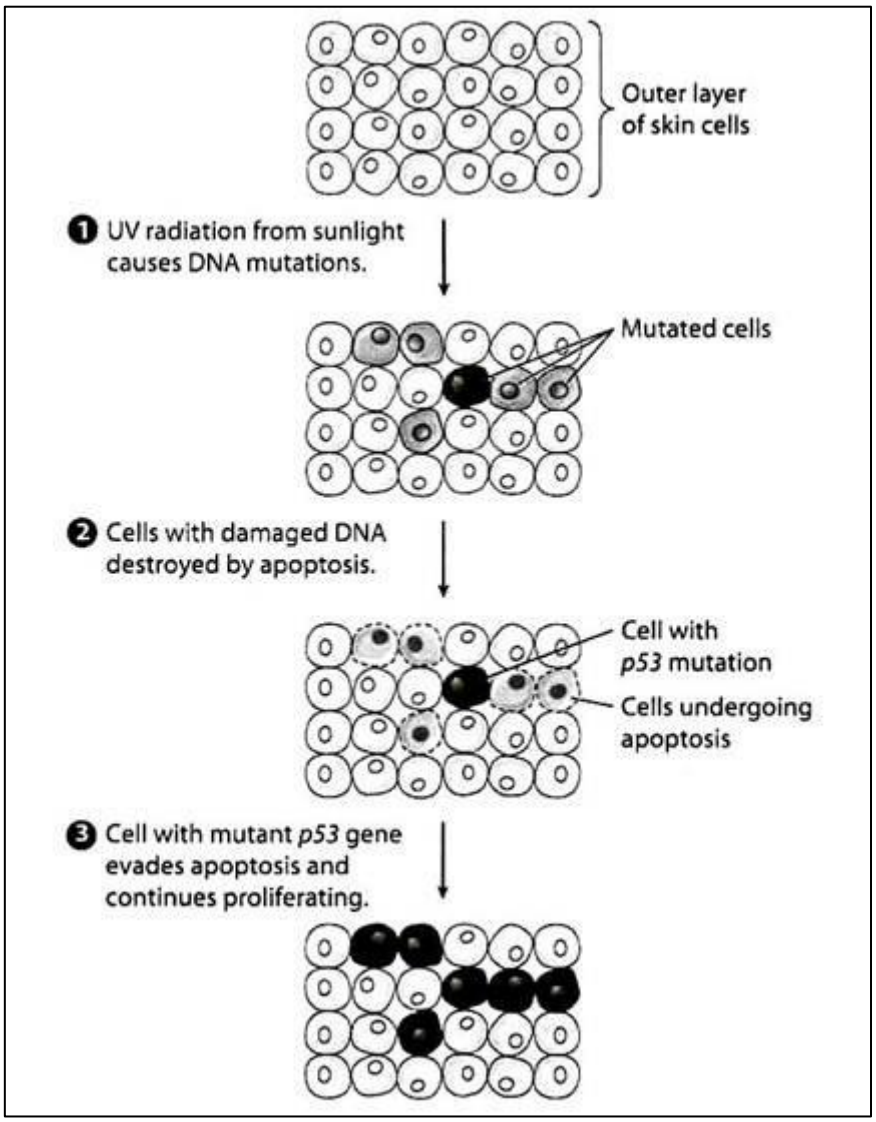


Fig 7: Sunlight-induced p^{53} mutations and the development of skin cancer

Sunlight-induced mutation of the p53 gene is thus comparable to the ini-tiation stage of chemical carcinogenesis, in which an initial mutation creates a precancerous cell that is later converted into a tumour by a promotion phase involving sustained cell proliferation.

Ionizing Radiation and Cancer

Although UV radiation is responsible for more cases of cancer than all other carcinogens combined, its inability to penetrate very far into the body means that it only causes skin cancer, which is often easy to cure.

We now turn our attention to higher-energy forms of radiation that penetrate into the body and can therefore cause cancer to arise in internal organs. This type of radiation is called ionizing radiation because it removes electrons from biological molecules, thereby generating highly reactive ions that damage DNA in various ways.

X-Rays Penetrate through Body Tissues and Cause Cancers of Internal Cells and Organs

In 1895, the first form of ionizing radiation that would turn out to cause cancer in humans was accidentally discovered by Wilhelm Roentgen, a German physicist. Roentgen was passing an electric current through a partially evacuated glass tube, called a cathode-ray tube, when he noticed that a fluorescent screen located across the room began to glow. Even after he covered the cathode-ray tube with black paper and moved it to another room, the screen glowed when the cathode-ray tube was turned on. Most astonishing, however, was the discovery that an image of the bones in Roentgen's hand appeared on the screen when he placed his hand between the cathode-ray tube and the screen. Radiation exhibiting such unusual properties was completely unknown at that time, so Roentgen named it X-rays. In recognition of the importance of this discovery, Roentgen was awarded the first Nobel Prize in Physics in 1901.

X-rays are a type of electromagnetic radiation exhibiting a wavelength shorter than that of UV radiation (see Fig 4). Because of their short wavelength, X-rays are highly energetic and will pass through many materials that cannot be penetrated by UV radiation, visible light, or other weaker forms of electromagnetic radiation. This is the property that allows X-rays to be used for viewing the inside of objects such as the human body.

Shortly after X-rays were discovered in 1895, newspaper headlines proclaimed "new light sees through flesh to bones!" and X-ray studios were opened around the country so that people could have "bone portraits" taken of themselves, even if they had no health problems! And doctors, of course, quickly embraced the new tool, which was to revolutionize many aspects of medical diagnosis and treatment. Unfortunately, medical practitioners and researchers were slow to recognize the hazards of X-rays. An early danger signal came from the laboratory of Thomas Edison, whose research technician routinely tested X-ray equipment by using it to take pictures of his own hands. The technician soon developed severe radiation burns and cancer arose in the burned tissue. Although both his arms were subsequently amputated, he died of metastatic cancer in 1904, the first cancer fatality attributed to X-rays. More cancers appeared in the next few decades as doctors specializing in the use of X-rays (radiologists) began to develop leukaemia at rates several times higher than normal. The suspicion that X-rays were causing these cancers was eventually confirmed by animal studies, which showed that animals exposed to X-rays develop cancer at rates that are directly proportional to the dose of radiation received (Figure 7). The risk of leukaemia is especially elevated, but X-rays pose a cancer threat to almost every tissue of the body.

There are three main forms of **nuclear radiation**, known as alpha (α), beta (β), and gamma (γ) radiation. Alpha and beta radiation both involve streams of charged particles of matter. Alpha particles are positively charged entities composed of two neutrons plus two protons (the nucleus of one helium atom); beta particles are electrons and therefore exhibit a negative charge.

In addition to these particulate forms of nuclear radiation, some radioactive elements emit gamma rays, which are a type of electromagnetic radiation and therefore exhibit no mass or charge. The wavelength of gamma rays is shorter than that of X-rays, making them the most energetic form of electromagnetic radiation (see Fig 4) radioactive Elements Emit Alpha, Beta, and Gamma Radiation:

Ionizing Radiation Initiates Carcinogenesis by Causing DNA Damage

As was the case for carcinogenic chemicals and UV radiation, DNA damage lies at the heart of the mechanism by which ionizing radiation causes cancer. The ability of ionizing radiation to trigger mutations was first described in the 1920s by Hermann Muller in studies involving fruit flies. When

the mutation rate is plotted against the dose of ionizing radiation, the dose-response curve appears to be linear over a wide range of radiation doses.

In contrast to UV radiation, which creates a distinctive type of DNA mutation (pyrimidine dimers), ionizing radiation damages DNA in a variety of ways (Figure 11). By definition, ionizing radiation strips away electrons from molecules, generating highly unstable ions that rapidly undergo chemical changes and break chemical bonds. Because roughly 80% of the mass of a typical cell is accounted for by water molecules, many of the bonds broken by ionizing radiation reside in water.

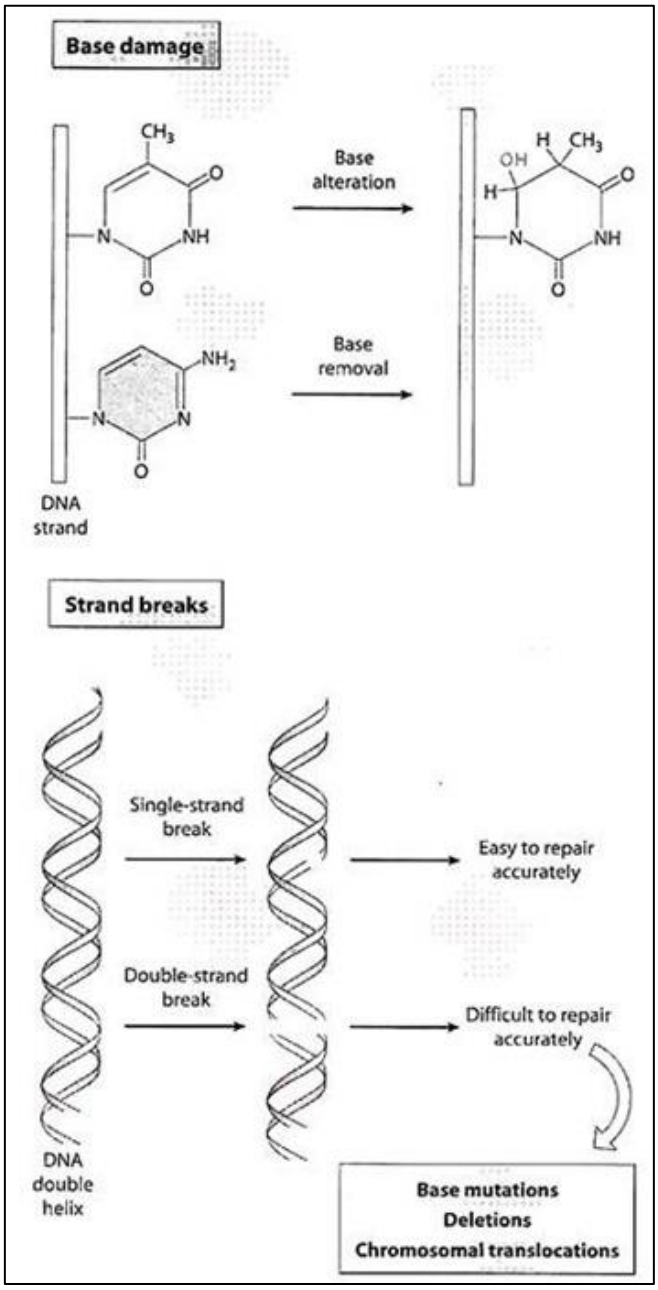


Fig 8: types of DNA damage caused by ionizing radiation

The disruption of water molecules produces highly reactive fragments called free radicals, a general term that refers to any atom or molecule containing an unpaired electron. The presence of an unpaired electron makes free radicals extremely reactive. One of the free radicals produced when ionizing radiation interacts with water is the hydroxyl radical (OH), which readily attaches itself to DNA

bases. The presence of these added hydroxyl groups alters the base-pairing properties of the bases during DNA replication, leading to various mutations. In addition to generating water-derived free radicals, ionizing radiation also attacks DNA directly, stripping away electrons and breaking bonds. Such reactions cleave the bonds that join bases to the DNA backbone, thereby causing individual bases to be lost; ionizing radiation also attacks the DNA backbone itself, creating single- or double-strand breaks in the DNA double helix.

Fortunately, it is relatively easy to repair single-strand breaks or the loss of individual bases because the opposite DNA strand of the double helix remains intact and serves as a template for fixing the defective strand by normal repair mechanisms.

Double-strand breaks are more difficult to fix, and imperfect attempts at repair may create localized mutations in the region of the break or larger-scale alterations, such as major deletions or sequence rearrangements. If double-strand breaks occur in more than one chromosome, DNA derived from two different chromosomes may be mistakenly joined together. The result is a chromosomal translocation in which a segment of one chromosome is physically joined to another chromosome.

It usually takes many years for cancer to arise following radiation-induced DNA damage. Radiation is thus acting in the initiation phase of carcinogenesis, playing a role comparable to that of mutagenic chemicals in the initiation of chemical carcinogenesis. As would be expected, treating radiation-exposed cells with promoting agents, such as phorbol esters, increases the rate at which tumors appear. Cells that have been initiated by exposure to ionizing radiation often exhibit a persistent elevation in the rate at which new mutations and chromosomal abnormalities arise. This condition, called genetic instability, creates conditions favourable for accumulation of the subsequent mutations that are required in the stepwise progression toward malignancy.

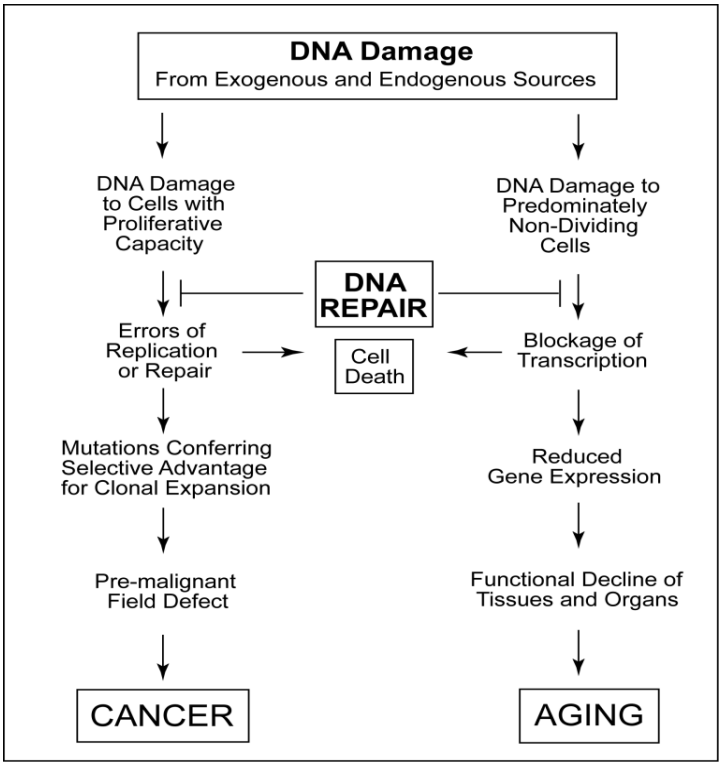


Fig 9: Role of DNA repair in carcinogenesis

The DNA sequence can be changed as the result of copying errors introduced by DNA polymerases during replication and by environmental agents such as mutagenic chemicals and certain types of

radiation. If DNA sequence changes, whatever their cause, are left uncorrected, both growing and nongrowing somatic cells might accumulate so many mutations that they could no longer function. In addition, the DNA in germ cells might incur too many mutations for viable offspring to be formed. Thus the correction of DNA sequence errors in all types of cells is important for survival.

The relevance of DNA damage and repair to the generation of cancer (carcinogenesis) became evident when it was recognized that all agents that cause cancer (carcinogens) also cause a change in the DNA sequence and thus are mutagens.

Probable questions:

1. Define chemical carcinogen with examples
2. Classify chemical carcinogens with examples.
3. Briefly discuss characteristics of chemical carcinogen.
4. Discuss the role of radiation in carcinogenesis.
5. Discuss the role of DNA damage repair in carcinogenesis.
6. State the role of ultraviolet radiation in carcinogenesis.
7. How mutations in the p53 Gene triggered by UVB Radiation lead to Skin Cancer?
8. How ionizing radiation initiates carcinogenesis by causing DNA damage?

Suggested readings:

1. Hardin, J. Bertoni, G and Klein smith, J. L. (2012). Becker's World of the Cell. 8th Edn, Pearson Benjamin Cummings, San Francisco.

2. Pal, A. (2011). Textbook of Cell and Molecular Biology 3rd Edn, Bokks and Allied, Kolkata.

3. Plopper, G, D. Sharp, Siroski, E (2015) Lewin's Cell 3rdEdition—Johns & Bartlett Publishers

4. Pollard and Earnshaw (2007). Cell Biology. 2nd. Edn Saunders.

5. Albert Bruce, Bray Dennis, Levis Julian ,Raff Martin, Roberts Keith and Watson James (2008).Molecular Biology of the Cell, V Edition, Garland publishing Inc., New York and London.

6. Cooper, G.M. and Hausman, R.E. (2009). The Cell: AMolecularApproach.5thEdition. ASM Press and Sunderland, Washington, D.C.; Sinauer Associates, MA.

Unit V

Cell-cell adhesion: types of cell binding, adhesive proteins, their role in cell-cell interaction

Objective: In this unit you will know about Cell-cell adhesion, types of cell binding, adhesive proteins and their role in cell-cell interaction.

Introduction

Cell adhesion is the process by which cells interact and attach to neighbouring cells through specialised molecules of the cell surface. Adhesion of cells is a primary feature of the architecture of many tissues.

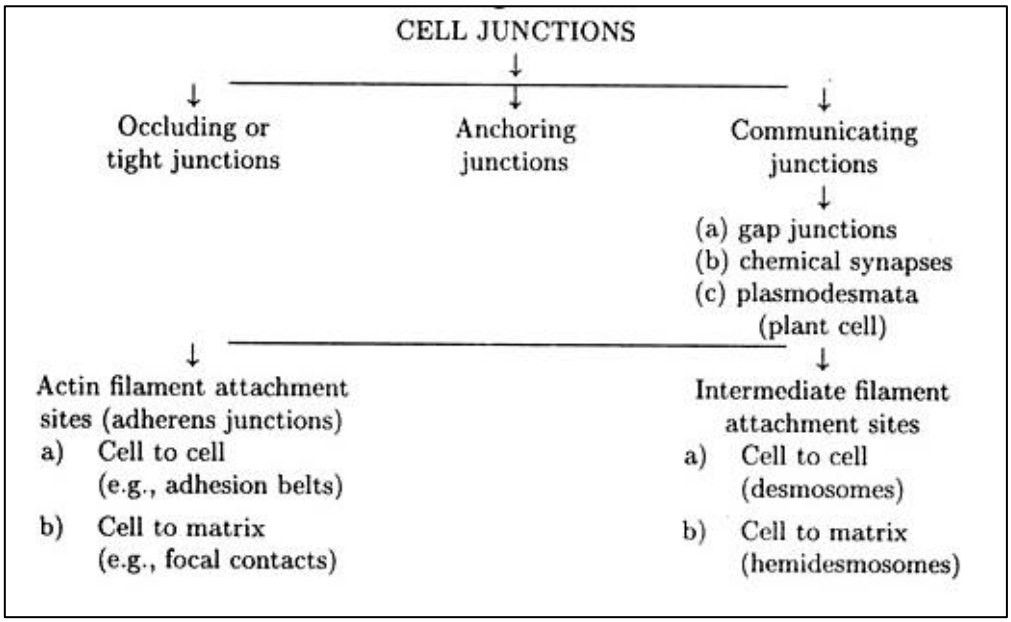
Cell junctions fall into three function groups:

1. Occluding or tight junction;

2. Anchoring junction;

3. Communicating junction.

The classification of all types of cell junctions is shown in details in the chart below.



1. Tight junction

Definition: Tight junctions are areas where the membranes of two adjacent cells join together to form a barrier. The cell membranes are connected by strands of transmembrane proteins such as claudins and occludins. Tight junctions bind cells together, prevent molecules from passing in between the cells, and also help to maintain the polarity of cells. They are only found in vertebrates, animals with a backbone and skeleton; invertebrates have septate junctions instead.

Structure: Tight junctions are a branching network of protein strands on the surface of a cell that link with each other throughout the surface of the membrane. The strands are formed by transmembrane proteins on the surfaces of the cell membranes that are adjacent to each other.

There are around 40 different proteins at tight junctions. These proteins can be grouped into four main types. Transmembrane proteins are wedged in the middle of the cell membrane and are responsible for adhesion and permeability. Scaffolding proteins organize transmembrane proteins. Signaling proteins are responsible for forming the tight junction and regulating the barrier. Regulation proteins regulate what proteins are brought to the cell membrane in vesicles.

Claudins and occludins are the two main types of proteins present at tight junctions, and they are both transmembrane proteins (Fig 1). Claudins are important in forming tight junctions, while occludins play more of a role in keeping the tight junction stable and maintaining the barrier between cells that keeps unwanted molecules out.

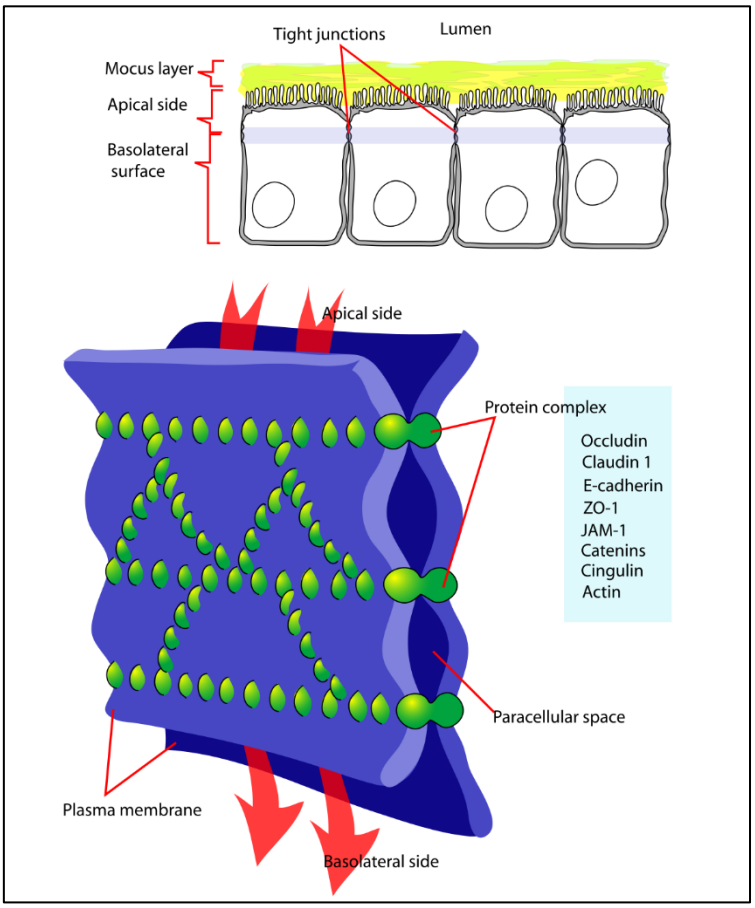


Fig 1: Cellular structure of tight junction

Function: Tight junctions have several different functions. Their most important functions are to help cells form a barrier that prevents molecules from getting through, and to stop proteins in the cell membrane from moving around. Tight junctions are often found at epithelial cells, which are cells that line the surface of the body and line body cavities. Not only do epithelial cells separate the body from the surrounding environment, they also separate surfaces within the body. Therefore, it is very important that the permeability of molecules through layers of epithelial cells is tightly controlled.

If molecules are blocked by tight junctions and physically unable to pass through the space in between cells, they must enter through other methods that involve entering the cells themselves. They could pass through special proteins in the cell membrane, or be engulfed by the cell through endocytosis. Using these methods, the cell has greater control over what materials it takes in and allows to pass through. However, in endothelial cells, certain proteins must be kept on certain sides of the cell. The apical, or outside layer, of the sheet of cells contains proteins that only let certain substances pass

through. The basal, or inside layer, is where cells let molecules pass through them by expelling them from their membrane in a process called exocytosis. Exocytosis also relies on specific proteins in order to work correctly. Tight junctions keep the correct proteins on the correct sides of the cell in order for these functions to occur. This also helps maintain the polarity of cells.

Another function of tight junctions is simply to hold cells together. The branching protein strands of tight junctions link adjacent cells together tightly so that they form a sheet. These strands are anchored to microfilaments, part of the cell's cytoskeleton that is made up of long strands of actin proteins. Microfilaments are located inside the cell, so the combination of microfilaments and sealing strands anchors the cells together from the inside and the outside.

Example: *The Epithelia of the Human Lung*

A report by Vermeer, et al., in the 20 March 2003 issue of Nature provides a striking example of the role of tight junctions.

- The epithelial cells of the human lung express a growth stimulant, called heregulin, on their apical surface and
- Its receptors on the basolateral surface. (These receptors also respond to epidermal growth factor (EGF), and mutant versions have been implicated in cancer.

As long as the sheet of cells is intact, there is no stimulation of its receptors by heregulin thanks to the seal provided by tight junctions. However, if the sheet of cells becomes broken, heregulin can reach its receptors. The result is an autocrine stimulation of mitosis leading to healing of the wound.

Several disorders of the lung

- the chronic bronchitis of cigarette smokers
- asthma
- cystic fibrosis

2. Adherens junctions

Adherens junctions provide strong mechanical attachments between adjacent cells.

- They hold cardiac muscle cells tightly together as the heart expands and contracts.
- They hold epithelial cells together.
- They seem to be responsible for contact inhibition.
- Some adherens junctions are present in narrow bands connecting adjacent cells.
- Others are present in discrete patches holding the cells together.

Adherens junctions connect bundles of actin filaments from cell to cell or from cell to extracellular matrix.

(i) Cell to Cell Adherens Junctions:

They are generally found at the interface between lateral plasma membranes of adjacent columnar epithelial cells, just below the region of the tight junctions. In the junctional zone, the intercellular space is filled with fine filaments.

They are connected with actin filaments and form a continuous band that girdles the inner surface of the plasma membrane of the connecting cells. This band is known as adhesion belt or zonula adherens and is' made of a web of 6 nm actin microfilaments.

The actin bundles attach to plasma membranes through a complex of intracellular attachment proteins containing vinculin. It is thought that the contractile actin filament bundles play an important role in

animal morphogenesis. They help in rolling up of the epithelial sheet into tube or other related structures.

(ii) Cell to Matrix Adherens Junctions:

The bundle of actin filaments within each cell comes out partly as trans membrane linker through some discrete sites of plasma membrane at the intracellular space and adhere the cell tightly with the extracellular matrix. In the junctional zone the specialised regions of plasma membrane are called focal contacts or adhesion plaques.

Adherens junctions are built from:

- **cadherins** — transmembrane proteins (shown in red) whose
    - extracellular segments bind to each other and
    - whose intracellular segments bind to
- **catenins** (yellow). Catenins are connected to actin filaments

There are 80 different types of cadherins. In most cases, a cell expressing one type of cadherin will only form adherens junctions with another cell expressing the same type. This is because molecules of cadherin tend to form homodimers not heterodimers.

Inherited mutations in a gene encoding a cadherin can cause stomach cancer. Mutations in a gene (*APC*), whose protein normally interacts with catenins, are a common cause of colon cancer.

Loss of functioning adherens junctions may accelerate

- the edema associated with sepsis;
- tumor metastasis.

Communicating Junctions:

It is a type of cell junction that mediates the passage of chemical or electrical signals from one interacting cell to its partner.

3. Gap junction

Definition: Gap junctions are a type of cell junction in which adjacent cells are connected through protein channels. These channels connect the cytoplasm of each cell and allow molecules, ions, and electrical signals to pass between them. Gap junctions are found in between the vast majority of cells within the body because they are found between all cells that are directly touching other cells. Exceptions include cells that move around and do not usually come into close contact with other cells, such as sperm cells and red blood cells. Gap junctions are only found in animal cells; plant cells are connected by channels called plasmodesmata instead.

Structure: In vertebrate cells, gap junctions are made up of connexin proteins (The cells of invertebrates have gap junctions that are composed of innexin proteins, which are not related to connexin proteins but perform a similar function.) (Fig 2). Groups of six connexins form a connexon, and two connexons are put together to form a channel that molecules can pass through. Other channels in gap junctions are made up of pannexin proteins. Relatively less is known about pannexins; they were originally thought only to form channels within a cell, not between cells. Hundreds of channels are found together at the site of a gap junction in what is known as a gap junction plaque. A plaque is a mass of proteins.

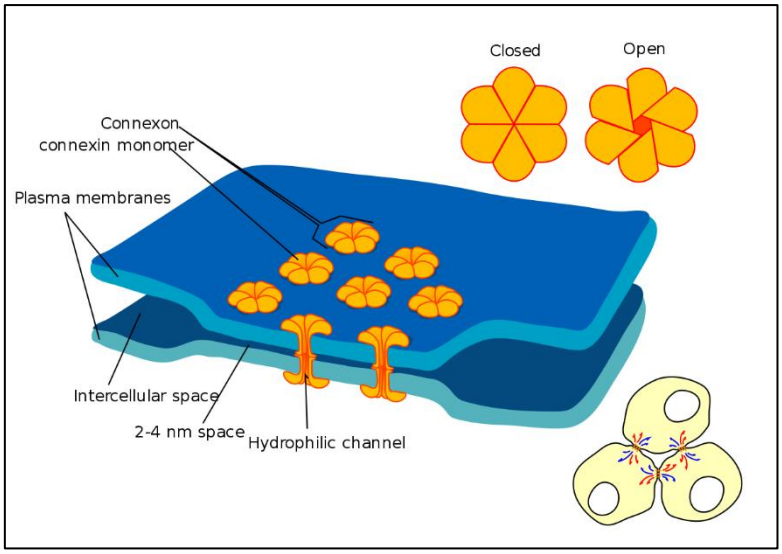


Fig 2: Structure of gap junction

Function: The main function of gap junctions is to connect cells together so that molecules may pass from one cell to the other. This allows for cell-to-cell communication, and makes it so that molecules can directly enter neighbouring cells without having to go through the extracellular fluid surrounding the cells. Gap junctions are especially important during embryonic development, a time when neighbouring cells must communicate with each other in order for them to develop in the right place at the right time. If gap junctions are blocked, embryos cannot develop normally.

Gap junctions make cells chemically or electrically coupled. This means that the cells are linked together and can transfer molecules to each other for use in reactions. Electrical coupling occurs in the heart, where cells receive the signal to contract the heart muscle at the same time through gap junctions. It also occurs in neurons, which can be connected to each other by electrical synapses in addition to the well-known chemical synapses that neurotransmitters are released from.

When a cell starts to die from disease or injury, it sends out signals through its gap junctions. These signals can cause nearby cells to die even if they are not diseased or injured. This is called the "bystander effect", since the nearby cells are innocent bystanders that become victims. However, sometimes groups of adjacent cells need to die during development, so gap junctions facilitate this process. In addition, cells can also send therapeutic compounds to each other through gap junctions, and gap junctions are being researched as a method of therapeutic drug delivery.

Examples:

- The action potential in heart (cardiac) muscle flows from cell to cell through the heart providing the rhythmic contraction of the heartbeat.
- At some so-called electrical synapses in the brain, gap junctions permit the arrival of an action potential at the synaptic terminals to be transmitted across to the postsynaptic cell without the delay needed for release of a neurotransmitter.
- As the time of birth approaches, gap junctions between the smooth muscle cells of the uterus enable coordinated, powerful contractions to begin.

Several inherited disorders of humans such as

- certain congenital heart defects and
- certain cases of congenital deafness have been found to be caused by mutant genes encoding connexins.

4. Chemical Synapse:

Neurological impulses are transmitted from neurons to target cell by the synapse. There are two types of synapse—chemical and electrical which differ in both structure, and function, In chemical synapse, a narrow region, the synaptic cleft separates the plasma membranes of the presynaptic and postsynaptic cells (Fig 3).

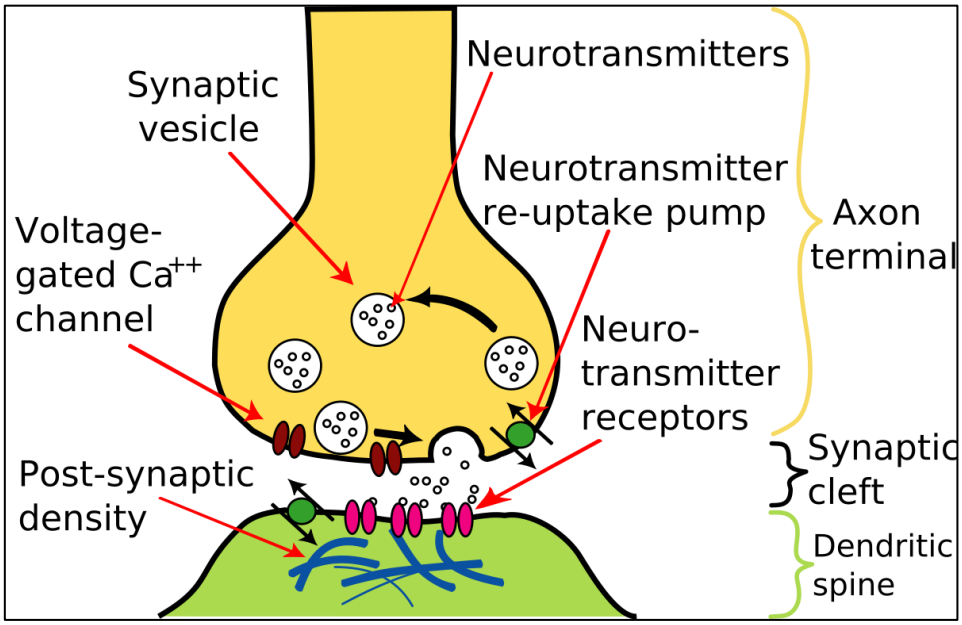


Fig 3: Structure of chemical synapse

The axon terminal of the presynaptical cell is filled with a particular neurotransmitter substance such as epinephrine or acetylcholine. The postsynaptic cells may be a dendrite, the cell body, the axon of another neuron or muscle or gland cell. When the postsynaptic cell is a muscle cell, the synapse is called neuromuscular junction or motor end plate.

When a nerve impulse reaches the axon terminal, some of the synaptic vesicles fuse with the membrane and are exocytosed and discharge its neurotransmitter contents into the cleft. The transmitter diffuses across the cleft and, after a lag period of about 0.5 millisecond binds to receptors on the postsynaptic cells.

The receptors fall into two categories: channel-linked receptors and non-channel linked receptors. The channel linked receptors, upon binding neurotransmitter, promptly change their conformation to create an open channel for specific ions to cross the membrane.

Therefore, they alter the membrane permeability. In case of non-channel receptors, the neurotransmitter-binding site is functionally coupled to an enzyme which catalyzes the production of an intracellular messenger, such as cyclic AMP, in presence of neurotransmitter. The intracellular messenger, in turn, causes changes in the postsynaptic cell, including modifications of the ion channels in its membrane.

Neurons communicating by an electric synapse are connected by gap junctions across which electric impulse can pass directly from the presynaptic cell to the postsynaptic one. Electric synapse allow an action potential to be generated in the postsynaptic cell with greater certainty than chemical synapses and without a lag period.

5. Plasmodesmata

Although each plant cell is encased in a boxlike cell wall, it turns out that communication between cells is just as easy, if not easier, than between animal cells. Fine strands of cytoplasm, called plasmodesmata, extend through pores in the cell wall connecting the cytoplasm of each cell with that of its neighbours.

Plasmodesmata provide an easy route for the movement of ions, small molecules like sugars and amino acids, and even macromolecules like RNA and proteins, between cells. The larger molecules pass through with the aid of actin filaments.

Structure:

- They are roughly cylindrical, membrane-lined channels with a diameter of 20 to 40 nm (Fig 4).
- They are constructed of three main layers, the plasma membrane, the cytoplasmic sleeve, and the desmotubule.
- Running from cell to cell through the center of most plasmodesmata is a narrower cylindrical structure, the desmotubule, which remains, continuous with elements of the SER membranes of each of the connected cells.
- Between the outside of the desmotubule and the inner face of the cylindrical plasma membrane is an annulus of cytosol, which often appears to be constricted at each end of the plasmodesmata.
- These constrictions may regulate the flux of molecules through the annulus that joins the two cytosols.
- The plasma membrane portion of the plasmodesma is a continuous extension of the cell membrane or plasmalemma and has a similar phospholipid bilayer structure.

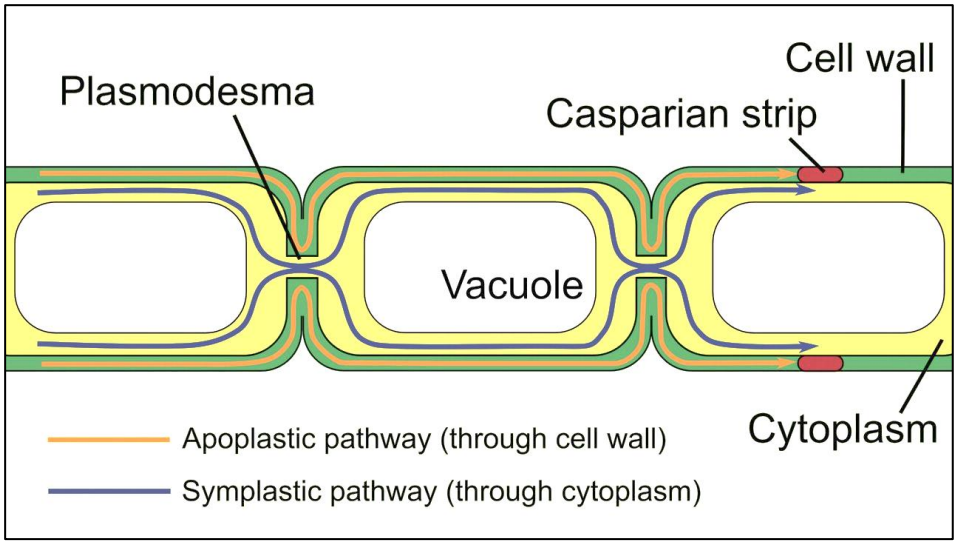


Fig 4: Structure of plasmodesmata

Function: They are narrow channels that act as intercellular cytoplasmic bridges to facilitate communication and transport of materials between plant cells. They serve to connect the symplastic space in the plant and are extremely specialized channels that allow for intercellular movement of water, various nutrients, and other molecules.

- Plasmodesmata function in intercellular communication, i.e., they allow molecules to pass directly from cell to cell.
- It has been suggested that plasmodesmata mediate transport between adjacent plant cells, much as gap junctions of animal cells. They allow the passage of molecules with molecular weights of less than 800 daltons.
- Plasmodesmata have been shown to transport proteins (including transcription factors), short interfering RNA, messenger RNA, viroids, and viral genomes from cell to cell.
- Plasmodesmata are also used by cells in the phloem, and symplastic transport is used to regulate the sieve-tube cells by the companion cells

6. Desmosomes

Desmosomes are button-like points of intercellular contact that rivet cells together. They are connected with intermediate filaments (a type of cytoplasmic filament 8-12 nm in diameter). The particular type of intermediate filaments joined to the desmosome depends on the cell type.They are keratin filaments in most epithelial cell, desmin filaments in heart muscle cells and vimentin filaments found in some of the cells covering the surface of the brain.

The structure of desmosome is very complex. On the cytoplasmic surface of each interacting plasma membrane there is a dense disc-shaped plaque (0.5 µm. in diameter) composed of mixture of intracellular attachment proteins called desmoplakins. Each plaque is connected with a thick network of intermediate filaments which pass along the surface of the plaque. Trans-membrane linker glycoproteins called desmogleins bind to the plaque and interact through their extracellular part to hold the adjacent membrane

7. Hemidesmosome

Hemi desmosomes or half-desmosomes are more or less morphologically similar to desmosomes but they are distinct from each other. Instead of joining adjacent cell membranes, hemi desmosome bind the basal surface of the cell to the underlying basal lamina. Both desmosomes and hemi desmosomes act as rivets to distribute tensile or shearing forces through an epithelium and its underlying connective tissue.

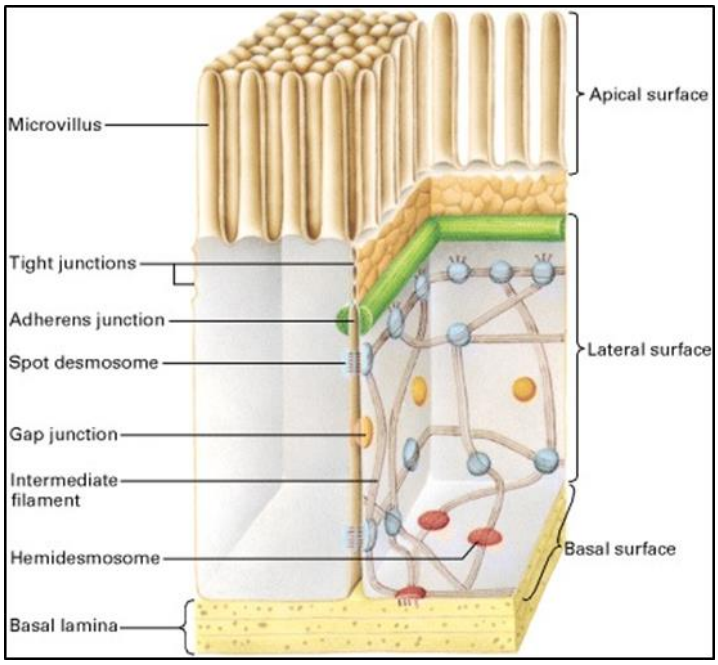


Fig: Schematic diagram of epithelial cells lining the small intestine and the principal types of cell junctions that connect them

Adhesive proteins, their role in cell-cell interaction

Cell-cell adhesion is controlled by cell adhesion molecules (CAMs) which recognize different ligands at cell junctions. Cell adhesion molecules (CAMs) are a subset of cell adhesion proteins located on the cell surface involved in binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion. In essence, cell adhesion molecules help cells stick to each other and to their surroundings. CAMs are uniformly distributed along the regions of plasma membranes that contact other cells, and the cytosol-facing domains of these proteins are usually connected to elements of the cytoskeleton. Eukaryotes, prokaryotes and viruses have disparate cell adhesions molecules. For mammalian cells there are four main classes of cell adhesion molecule (Fig 5):

1. Cadherins (calcium dependent glycoproteins)
2. Integrins (transmembrane receptor proteins not dependent on calcium)
3. Immunoglobin superfamily members (molecules involved in cell adhesion with an immunoglobin domain that are not dependent on calcium)
4. Selectins (single-chained glyoproteins that are calcium dependent)
5. Connexins

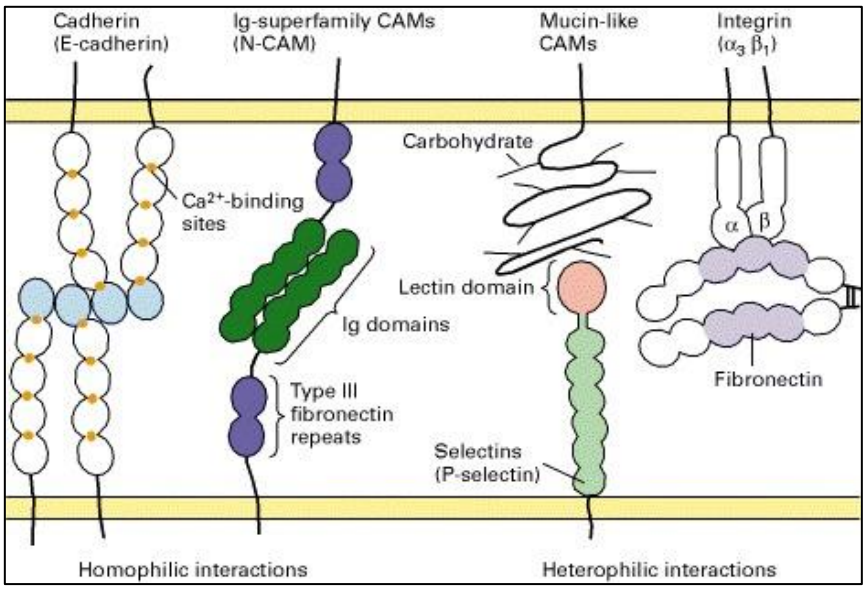


Fig 5: Major families of cell-adhesion molecules (CAMs)

Integral membrane proteins are built of multiple domains. Cadherin and the immunoglobulin (Ig) superfamily of CAMs mediate homophilic cell-cell adhesion. In a heterophilic interaction, the lectin domain of selectins binds carbohydrate chains in mucin-like CAMs on adjacent cells in the presence of Ca^{2+}.

Cell-cell recognition and cell-cell adhesion

Cell-cell recognition is an important part of the mechanism behind cell-cell adhesion. An early study noted the affinity found in cell adhesion molecules through the observation that cells from the same tissue preferentially adhere to one another. Cells disassociated from two separate organs can form a pellet when mixed, which gradually separate into cells derived from the same organ over time.

There are over one hundred different types of vertebrate cadherins and the experiment has been repeated with mixed cells sorted via cadherin type. It is these adhesive properties that allow for stable

tissue architecture, albeit more recent assays have observed heterotypic binding affinity. It is believed that kinetic specificity allows cell adhesion molecules to recognize each other rather than broader thermodynamic specificity.

Types of Adhesion molecules

1. Cadherins

- The cadherin superfamily comprises classical and non-classical cadherins
- Present in all multicellular animals
- Mediate calcium ion (Ca^{2+}) dependent cell-cell adhesions
- more than 180 members in humans
- *Classical cadherins*
    - (e.g.: E-cadherin, N-cadherin and P-cadherin) contain 5 cadherin repeats
- *Require calcium ions to bind*
- Homophilic binding through end element
- Functional unit a dimer
- *Non-classical cadherins*
    - (e.g. desmosomal cadherin, protocadherins and T-cadherins) are more distantly related in sequence
- Varying number of cadherin repeats
- Some non-classical cadherins have primarily a signalling function

Structure: A cadherin generally has a cytoplasmic domain, a transmembrane domain, and an extracellular domain (Fig 6). The latter is comprised of five subdomains held together by calcium ions. Removal of these ions will result in the collapse of the extracellular domain, thus disrupts homophilic adhesion. This is why cadherins are described as calcium-dependent cell adhesion molecules.

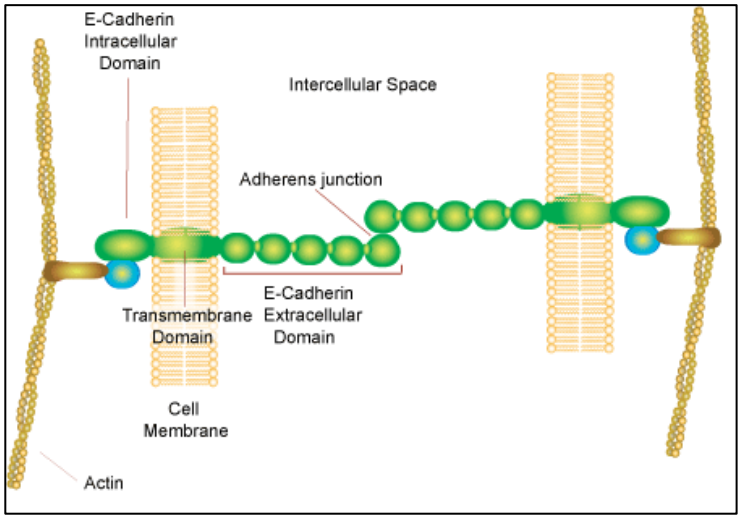


Fig 6: Structure of cadherin protein

2. Immunoglobulin Superfamily

- Vertebrates have 100+
- In addition to adhesion they also have role in immune system
- Contain varying number of Ig-related domains
- G. Edelman - Nobel Prize in Physiology or Medicine in 1972
- "for their discoveries concerning the chemical structure of antibodies"
- studying the nervous system

3. Selectins

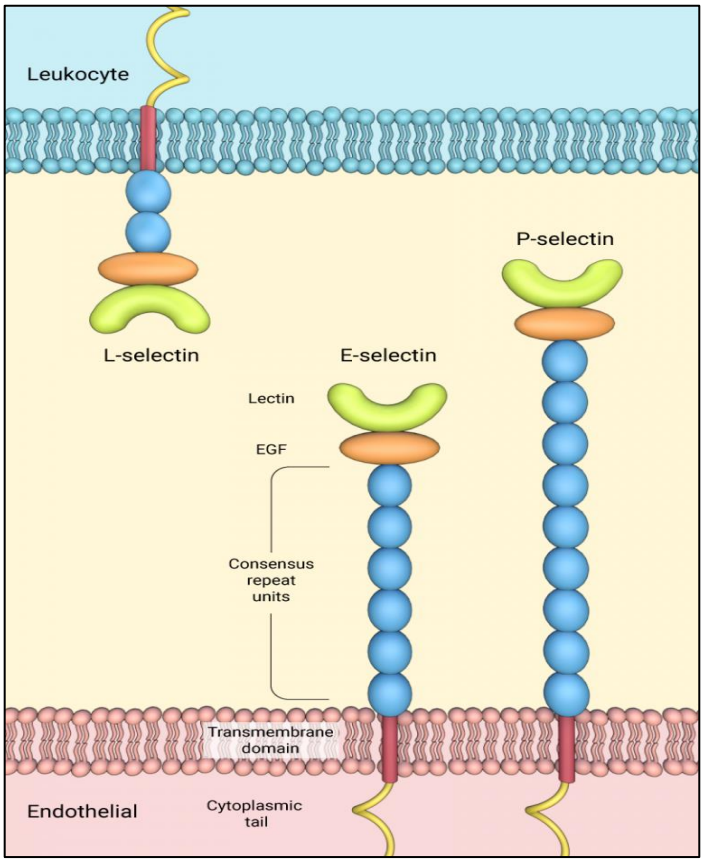


Fig 7: Selectin structure

- Cell Surface carbohydrate-binding proteins
- Vertebrates have only in circulatory system
- Role in inflammatory response: adhesion of leukocytes (blood cells) to endothelium (vessel wall)
- Cooperate with integrins and Ig-SF receptors
- Selectins 2 Heterophilic interactions
- Bind counterreceptors
- L-selectin on white blood cells (Fig 7)
- P-selectin on blood platelets and on endothelial cells that have been locally activated
- E-selectin on activated endothelial cells

4. **Integrins** (Fig 8)

- Mammals have genes for 18 alpha and 8 beta integrins
- Role in cell adhesion to extracellular matrix (ECM) basement membranes
- Induction of cell polarization by adhesion
- Glycosylated proteins
- Bind through C terminal lectin domain of selectin
- Comprising sandwich of beta sheets
- Held together by hydrophobic interactions
- Mainly receptors for ECM proteins
- Fibronectin, laminin, collagen
- Some heterotypic binding Ig superfamily
- Interact with cell cytoskeleton

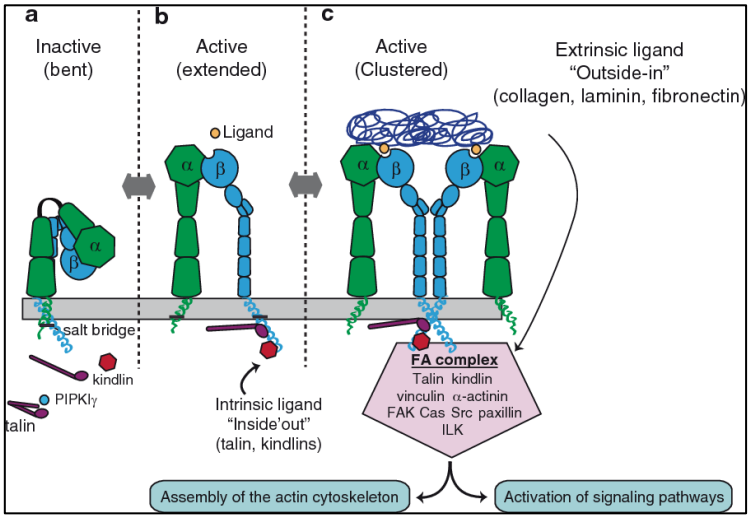


Fig 8: Structure of integrin

5. Connexins

Connexins are tetratransmembrane proteins that assemble into hexameric pore-forming structures known as connexons or hemichannels. Connexons typically dock with their counterparts in adjacent cells to form intercellular gap junction channels, but may remain unpaired as cell surface hemichannels.

Structure:

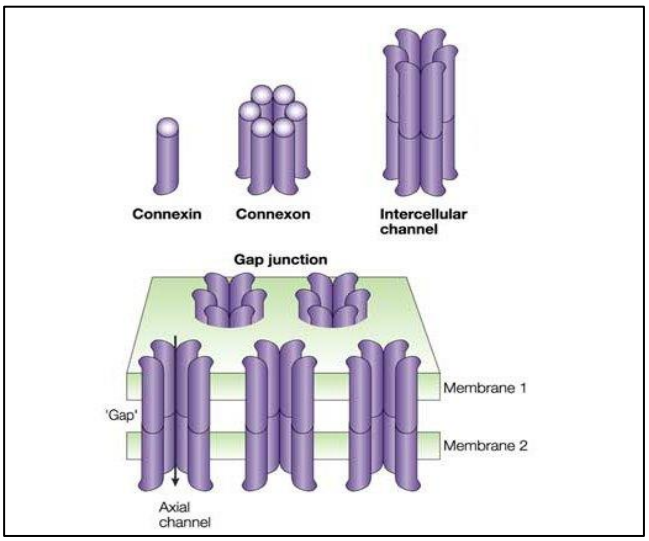


Fig 9: Structure of connexins

Connexins contain four highly ordered transmembrane segments (TMSs), primarily unstructured C and N cytoplasmic termini, a cytoplasmic loop (CL) and two extra-cellular loops, (EL-1) and (EL-2). Connexins are assembled in groups of six to form hemichannels, or connexons, and two hemichannels then combine to form a gap junction.

Function:
Connexin gap junctions are found only in vertebrates, while a functionally analogous (but genetically unrelated) group of proteins, the innexins, are responsible for gap junctions in invertebrate species. Innexin orthologs have also been identified in Chordates, but they are no longer capable of forming gap junctions. Instead, the channels formed by these proteins (called pannexins) act as very large transmembrane pores that connect the intra- and extracellular compartments.

Within the CNS, gap junctions provide electrical coupling between progenitor cells, neurons, and glial cells. By using specific connexin knockout mice, studies revealed that cell coupling is essential for visual signaling. In the retina, ambient light levels influence cell coupling provided by gap junction channels, adapting the visual function for various lighting conditions. Cell coupling is governed by several mechanisms, including connexin expression

Importance of cadherins for cell-cell adhesion

The calcium dependent cell adhesion molecules cadherins are usually 700-750 amino acids long. They are utilized in both desmosomes and adherens junctions to form protein complexes for cell-cell attachment. In desmosome complexes, a bridge is formed between the cadherins of each cell through homophilic binding.

This particular protein complex provides a strong adhesion useful for providing mechanical strength; therefore, desmosome complexes are abundant in heart and epidermis tissue that are frequently subjected to mechanical stress.

For adherens junctions, cadherins interact with actin filaments through intermediate anchor proteins called catenins. This type of cell-cell adhesion is common in epithelial and endothelial tissues. Calcium has a pivotal role in cadherin functional activity by maintaining the rigid structure required for binding. In short, the calcium ions produce an inflexible structure through their positioning between each pair of cadherins, while the rigidity of the structure is positively correlated with the amount of calcium ions available.

Selectins and cell-cell adhesion

Selectins are another type of calcium-dependent cell adhesion molecules that allow cell-cell interactions within the blood stream. Different types of selectin are utilized for white blood cells, blood platelets and endothelial cells.

The cell-cell adhesions within the bloodstream are formed through the selectin binding to a specific oligosaccharide on the adjacent cell. The binding of white blood cells to the endothelial cells lining blood vessels is particularly important in allowing the transportation of cells from the blood stream into tissues.

This cell-cell interaction between white blood cells and endothelial cells is strengthened by integrins, another cell adhesion molecule. The combination of selectins and integrins provides the weak adhesion that allows white blood cells to roll along the surface of the blood vessel. However, when necessary, the two cell adhesion molecules can also bind the blood cell strongly to the endothelial cell surface for exit through the blood vessel between endothelial cells.

Adhesion mediated by selectins

Selectins are a family of specialised CAMs involved in transient cell–cell adhesion occurring in the circulatory system. They mainly mediate the movement of white blood cells (leukocytes) in the bloodstream by allowing the white blood cells to "roll" on endothelial cells through reversible bindings of selections. Selectins undergo heterophilic bindings, as its extracellular domain binds to carbohydrates on adjacent cells instead of other selectins, while it also require Ca^{2+} ions to function, same as cadherins. Cell–cell adhesion of leukocytes to endothelial cells is important for immune responses as leukocytes can travel to sites of infection or injury through this mechanism. At these sites, integrins on the rolling white blood cells are activated and bind firmly to the local endothelial cells, allowing the leukocytes to stop migrating and move across the endothelial barrier.

Adhesion mediated by members of the immunoglobulin superfamily

The immunoglobulin superfamily (IgSF) is one of the largest superfamily of proteins in the body and it contains many diverse CAMs involved in different functions. These transmembrane proteins have one or more immunoglobulin-like domains in their extracellular domains and undergo calcium-independent binding with ligands on adjacent cells. Some IgSF CAMs, such as neural cell adhesion molecules (NCAMs), can perform homophilic binding while others, such as intercellular cell adhesion molecules (ICAMs) or vascular cell adhesion molecules (VCAMs) undergo heterophilic binding with molecules like carbohydrates or integrins. Both ICAMs and VCAMs are expressed on vascular endothelial cells and they interact with integrins on the leukocytes to assist leukocyte attachment and its movement across the endothelial barrier.

Probable questions:

1. Write short note on tight junction with diagram.
2. Name the proteins present at tight junctions.
3. Discuss the structure and function of gap junction.
4. Discuss chemical synapse junction in details.
5. What do you mean by plasmodesmata and desmosome?
6. Describe the structure of plasmodesmata with suitable diagram.
7. What do you mean by hemi desmosome?
8. What do you mean by adhesive protein? Give example.
9. Write short notes on cadherin.
10. Draw the structure of selectin protein.
11. What is the function of connecxin?
12. Describe the role of adhesive proteins in details.

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Unit VI

Morphogenesis, differentiation, movement of leucocytes into tissues

Objective: In this unit you will know about morphogenesis, differentiation and movement of leucocytes into tissues.

Introduction

Morphogenesis means the generation of form, and usually in the context of developmental biology where it means the generation of tissue organization and shape in animal and plant embryos. Morphogenesis therefore deals with apparently straightforward problems such as: how epithelial ducts branch in glands, how nerves migrate to and recognize their targets, how mesenchymal cells come together to form pre-muscle and pre-bone condensations, how tendons link to the appropriate bones, and how cells change their shapes.

Morphogenesis also covers more complicated questions which involve several coordinated morphogenetic processes such as: how bones are shaped, and how the early mammalian heart tube reorganizes itself and its associated blood vessels to produce the 4-chambered adult heart. Indeed, it covers anything and everything to do with biological shaping and developmental anatomy. While the questions are straightforward, they have proven difficult to answer.

Morphogenesis is one of the four key interrelated classes of event that characterize all of development:

1. *Patterning*: The setting up of the positions of future events across space (at a variety of scales)
2. *Regulation of timing*: The 'clock' mechanisms that regulate when events happen. Clocks can directly regulate morphogenesis of individual tissues such as somites, and changes of relative timing of events (heterochrony) can drive the evolution of new body plans.
3. *Cell differentiation*: Changes in a cell's set of expressed genes (its molecular phenotype)
4. *Morphogenesis*: The processes that generate tissue organization and shape and are usually the downstream response to the timing and patterning.

Each of these processes usually involves signalling from one tissue to another, the signals resulting in changes in protein activity and in gene expression that generate events (motors of change) that can be either cell-autonomous or can involve cell cooperation.

"Morphogenesis is important"

- It is responsible for tissue organization and hence for much of an organism's anatomy, physiology and behaviour.
- Mutations that affect morphogenesis underpin many human congenital abnormalities.
- Mutations that alter shape alter the fitness of a species under selection pressure and so drive evolutionary change.

"Morphogenesis is difficult to study"

Current knowledge about the morphogenesis of complex tissues is limited for three reasons:

1. Many of the key events take place during early development when organ rudiments are small and difficult to study, although genetic manipulation is now allowing morphogenesis to be investigated in organisms such as *Drosophila* with very small embryos.
2. Most tissues will not develop much of their form in vitro and so are inaccessible to standard experimental manipulation.
3. The intrinsic complexity of morphogenesis makes experimentation difficult.

Differentiation and morphogenesis represent two different terms that address the development of biological organisms. Differentiation refers to how cells become specialized, whereas morphogenesis refers to the development of the forms of living organisms.

Morphogenesis/Development of leukocytes

Monophyletic and Polyphyletic Theories

Broadly, there are two views regarding the development of all blood cells in postnatal life:

(a) The monophyletic theory, and

(b) The polyphyletic theory.

The monophyletic theory holds that all the blood cells are derived from a common primitive ancestor, which is called the 'stem cell' or haemocytoblast. The polyphyletic school, in its complete form, holds that for every variety of blood cell, there is a distinct type of 'blast cell; viz., erythroblast, myeloblast, lymphoblast, monoblast, etc. The dualistic school believes that there are two distinct types of primitive cells.

One of them remains in the bone marrow and gives rise to the red cells, granulocytes and megakaryocytes, from which the platelets develop. The other cell remains in the lymphoid tissue from which the lymphocytes are derived. Similarly, there is a trialistic school believing in three primitive cells. All school agrees that in the early embryo all the blood cells are derived from a single primitive reticulo-endothelial cell.

It is generally conceded that, in postnatal life, the development of leucocytes is completely extravascular. The granulocytes are derived exclusively from the red marrow; while the lymphocytes and the monocytes come mainly from the spleen and lymphatic glands and to some extent, from the bone marrow.

Development of Granular Leucocytes or Granulocytes:

1. Reticulum Cell of the Bone Marrow:

According to the monophyletic theory this stage starts with haemocytoblast

2. Primitive White Blood Cell:

Cells are large with a large number of round nucleus and a thin rim of basophilic, non-granular cytoplasm.

Myeloblast Smaller in size (12 to 18 μ), non-granular cytoplasm and a round or bean-shaped nucleus with several nucleoli. There are numerous mitochondria. They are non-motile or less motile. They divide and form the next stage. It should be noted that up to this stage the cytoplasm has no granules when stained with the ordinary Romanowsky methods.

By the peroxidase method granules may be found in the more mature of the myeloblast. The number of nuclei is an important differentiating point from lymphoblast. The latter have definite and distinct nucleoli whereas in the, former, the nucleoli look like irregular gaps

3. Myelocyte:

This stage is characterised by certain remarkable peculiarities which are quite distinct from the previous stages.

The peculiarities are as follows:

(a) Multiplicity although all the cells before this stage are multiplying, yet the multiplicity of the myelocytes is maximum. It is by the multiplication of the myelocytes that the normal supply of the white cells is kept up.

(b) Granules appear in the cytoplasm.

The granules may take neutral, acid or basic stains and accordingly the myeloytes are of three varieties:

(1) Neutrophilic myelocyte,

(2) Eosinophilic myelocyte,

(3) Basophilic myelocyte.

Recent observations show that each type of myelocyte passes through three stages of development—A, B, and C. Type A contains few granules and many mitochondria. Type C contains many granules but few mitochondria.

Type B is intermediate:

(c) Cytoplasm is less basophilic,

(d) Nucleoli disappear and chromatin is coarser.

4. Metamyelocyte:

At this stage, again certain characteristic changes appear:

(a) The nucleus becomes deeply indented and is almost bilobed. This shows that maturation is advancing,

(b) The cells no more multiply but only mature henceforward,

(c) Amoeboid movement appears and due to this, a few of these cells may burrow into the blood vessels, and

(d) Appear in the peripheral circulation.

5. Leucocyte:

The nucleus contains many lobes which are produced by maturation of the previous stage. The maturation takes place in the bone marrow. Because once in the peripheral circulation, no more maturation can take place. It is believed that the degree of maturation is proportional to the number of lobes of the nucleus.

i) Development of Lymphocytes:

In the central part of lymph node, there is a small area about 1 mm in diameter and pierced by a small blood vessel. This is called the germinal centre or secondary lymphoid follicle. It takes a lighter stain

than the surrounding area. Some of the reticulum cells in this area proliferate and give rise to the lymphoblast.

In recent years, evidence has been accrued and shows that the thymus is an important and probably the main source of production of lymphocytes. Apparently, there seem to be two types of lymphocytes in the peripheral blood, a longer-lived type derived from the thymus, and a shorter-lived type, probably arising from the lymphoid tissue.

The stages of proliferation are the same. The lymphoblast is 15 to 20 μ in diameter, with a round or oval nucleus and a non-granular cytoplasm. These cells divide and give rise to the large lymphocyte. The normal number of lymphocytes in the blood is maintained chiefly by the division of the lymphoblast cells.

The large lymphocytes are fairly mature cells and do not multiply any further. These cells are found in the circulation. The small lymphocytes are derived by further maturation of the large lymphocytes. These cells leave the gland through the efferent lymphatics, thoracic duct and right lymphatic duct, and reach the circulation.

ii) Development of Monocytes:

The monocytes are developed mainly from the reticulum cells of spleen and lymph nodes and to a lesser extent of the bone marrow.

The stages are: Reticulum cells → Monoblasts → Monocytes.

Monocytes can be differentiated from the myeloblast by positive peroxidase reaction in the latter. The so-called 'transitional leucocytes' are one variety of monocytes.

Differentiation of leucocytes

Inflammatory responses often involve the selective accumulation in tissues of complex mixtures of leukocytes. In order to understand the processes governing migration and accumulation of mature leukocytes, it is useful to begin by considering the development of leukocytes in adult bone marrow, as well as the earliest migrations made by hematopoietic cells during prenatal development.

In adult mammals, bone marrow is the direct or indirect source of all lineages of hematopoietic cells that mediate inflammation. These lineages arise from rare self-renewing, pluripotent hematopoietic stem cells (HSC) through a series of intermediate progenitors with restricted self-renewal capacity, restricted developmental potential, or both. These intermediate progenitors either differentiate to mature effector cells in the bone marrow, or leave the marrow and mature elsewhere.

Early Sites of Haematopoiesis

HSC are defined as cells individually capable of giving rise to all lineages of hematopoietic cells (erythrocytic, megakaryocytic, and numerous subsets of lymphocytic and myelomonocytic cells) as well as self-renewal. In mammals, the earliest haematopoiesis is extraembryonic, occurring in the yolk sac blood islands, which appear at embryonic day (E) 7.5 in the mouse. Yolk sac blood production is termed *primitive* haematopoiesis, in that mostly nucleated erythrocytes with fetal-type haemoglobin are made, in contrast to the enucleated erythrocytes with adult-type haemoglobin that arise from *definitive* haematopoiesis.

Early Migrations of Haematopoietic Stem Cells

HSC migration appears to occur at least twice during prenatal development, and both of these migrations are likely to be associated with HSC expansion. The first migration involves the

colonization of the fetal liver by hematopoietic cells; this may be accomplished by migration of HSC from the yolk sac or both, to the liver. In the case of migrations of HSC from aorta gonad-mesonephros (AGM) and / or yolk sac to liver, and from liver to spleen and bone marrow, cells may be mobilized in narrow windows of development. For this to be true, there must be tightly regulated changes in the adhesive and / or chemotactic properties of HSC, as well as mechanisms to direct their homing to specific microenvironments in target tissues.

Adult Hematopoietic Ontogeny

The bone marrow is the direct or indirect source of the entire daily output of hematopoietic cells in normal adult mammals. The productivity of the bone marrow is truly astounding; an estimated 1 X 1010 granulocytes are made each day in humans to replace senescing cells, along with 2 X 1011 erythrocytes and 4 X 1011 platelets. Some cells, such as erythrocytes and neutrophils, mature fully in the bone marrow prior to entering the circulation, whereas other cells, such as T and B lymphocytes, mature to varying degrees in extra medullary sites like the thymus, spleen, and lymph nodes.

The bone marrow presents obstacles to in situ study, in part because it is encased in bone, so gaining access to undisturbed tissue can be difficult, and also because bone marrow cells with vastly different functions and developmental potentials are often morphologically indistinguishable. For example, HSC resemble large lymphocytes, and can only be identified at present by analysing a sufficiently complex set of markers that in situ localization of HSC is precluded. Because of these obstacles, and because hematopoietic cells can be transplanted easily, more is known about the function of bone marrow cells in vitro, or in the setting of transplantation, than about in vivo bone marrow biology.

The Concept of the Hematopoietic Niche

Bone marrow has the daunting task of supporting partial or complete maturation of numerous cell lineages, including erythrocytes, megakaryocytes (platelet precursors), lymphocytes (B cells, T cells, and natural killer, or NK cells), and several types of granulocytes (Fig 1).

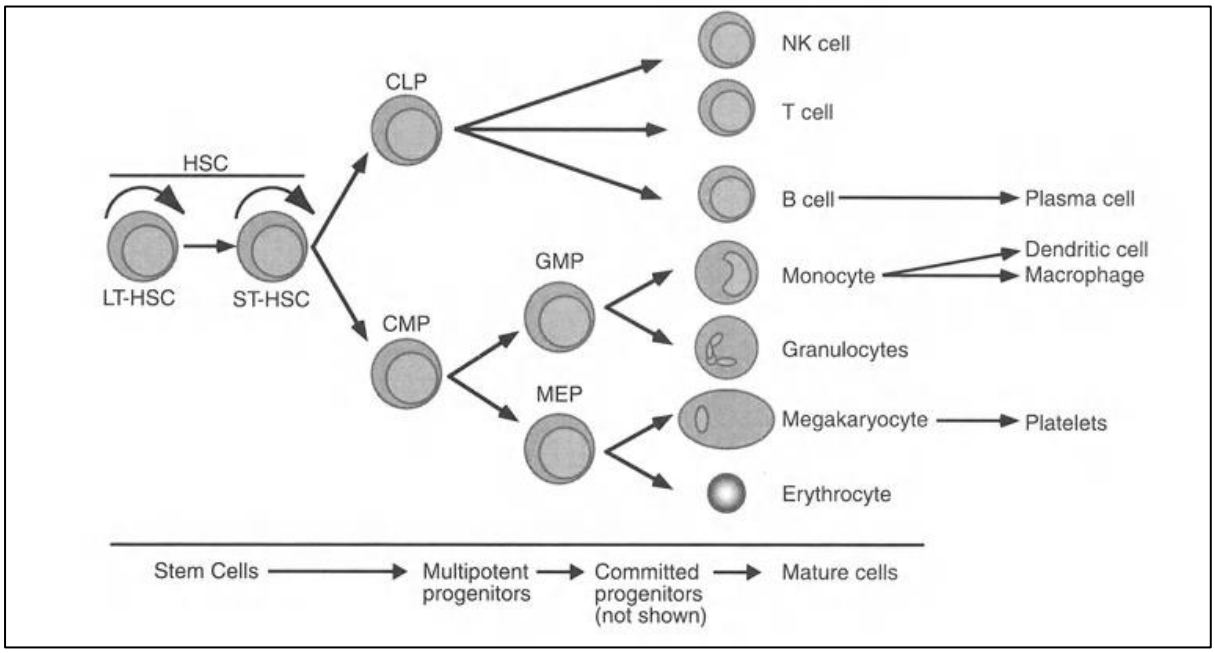


Fig 1. Hematopoietic ontogeny in adults: Long-term hematopoietic stem cells (LTHSC) can self-renew or differentiate. Differentiating LT-HSC become short-term (ST) HSC that produce multipotent progenitors. The self-renewal potential of LT-HSC exceeds the life span of the animal, whereas that

of ST-HSC is much more limited. Self-renewal in multipotent progenitors, if present, is minimal. Multipotent progenitors become committed progenitors that produce all lineages of mature cells; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/ macrophage progenitor; MEP, megakaryocyte/ erythroid progenitor; NK, natural killer.

The presumption that various lineages have special requirements for maturation has given rise to the concept that bone marrow is functionally divided into specialized microenvironments or "niches." In human bone marrow, for example, erythropoiesis occurs in small islands of cells that, at least superficially, appear to be zones specialized for erythropoiesis (granulopoiesis, however, is dispersed). Although myelopoiesis and erythropoiesis are sometimes observed in extra medullary locations, substantial levels of lymphomyeloid multiline age haematopoiesis cannot be sustained by tissues other than bone marrow. This is consistent with the notion that bone marrow contains specialized microenvironments.

Movement of leukocytes

Diapedesis of leukocytes is a major event in the migration of leukocytes from the blood circulation to sites of inflammation or tissue injury and in the recirculation of lymphocytes from the blood to the lymphatic compartment. Diapedesis of leukocytes is often confused with the terms ‘leukocyte transmigration’ or ‘leukocyte extravasation’. These terms define the entire process of leukocyte migration from the blood circulation to the extravascular connective tissue, across the vessel wall endothelium. Diapedesis is regarded as the final event in the transmigration, i.e. the actual penetration of the vascular endothelium and the sub endothelial matrix.

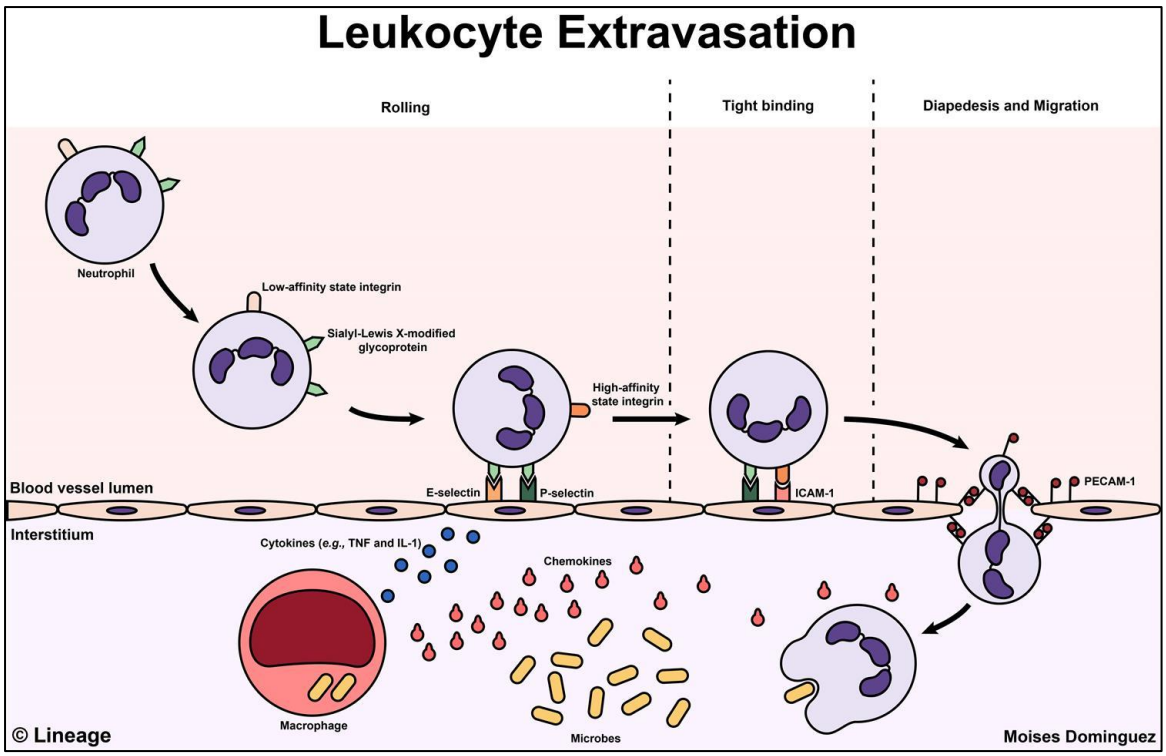
The inflammatory or leukocytic infiltrate consists of white blood cells which leave the blood and enter (infiltrate) the inflamed connective tissue.

Cells of the inflammatory infiltrate include neutrophils, lymphocytes and monocytes. Immigration of these cells into peripheral tissues is one of the principal purposes for inflammation, bringing to a site of injury the immune-system cells which can combat infection and clean up damaged tissue.

Neutrophils (neutrophilic leukocytes) are the first white blood cells to enter the tissue during acute inflammation. Neutrophils are anti-bacterial cells which lyse (break down) bacterial cells by releasing lysosomal enzymes. Neutrophils recognize bacteria as foreign by the antibody molecules which have attached to the bacterial surface. Antibody molecules (molecules which bind to one specific antigen or foreign substance which the body has previously encountered) are found in blood plasma and interstitial fluid.

Lymphocytes accumulate somewhat later during the inflammatory process. Their presence in large numbers indicates the continuing presence of antigen and thus may suggest an established infection. Lymphocytes produce the multitude of diverse antibody molecules (one specific type of antibody per lymphocyte) which provide the mechanism for chemical recognition of foreign materials (distinguishing between self and non-self) and so for mediating and regulating immune responses.

Monocytes are phagocytic cells which circulate in the blood. An equivalent cell type, called the *macrophage*, is a resident cell in connective tissue. Monocytes/macrophages engulf and digest foreign microorganisms, dead or worn-out cells, and other tissue debris. They interact closely with lymphocytes to recognize and destroy foreign substances.



- Leukocytes play an important role in eliminating offending agents
  - neutrophils and macrophages are capable of destroying microbes, necrotic tissue, and foreign substances
- The process of leukocytes migrating from the blood vessel to tissue involves multiple steps and are mediated by adhesion molecules and chemokines
  - margination
    - leukocytes become redistributed closer to the vessel wall
  - rolling
    - leukocytes transiently attach to endothelium and then detach
      - these cells therefore "roll" on the vessel wall
    - the endothelium possesses E-selectin and P-selectin
      - cytokines from the inflamed tissue regulate selectin expression
      - Sialyl-Lewis X protein binds to E-selectin and P-selectin (found on leukocytes)
  - firm endothelial adhesion
    - mediated by integrin proteins
      - vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule-1 (ICAM-1) are found on the endothelium
      - low affinity integrin are found on leukocytes
  - migration through the vessel wall
    - migration to tissue

Probable questions:

1. Define morphogenesis
2. What is patterning?
3. What is the importance of morphogenesis?
4. Give a detail discussion about the development of leukocytes.
5. Discuss the differentiation of leukocyte in details.
6. Write a short note on haematopoietic niche?
7. Discuss the movement of leucocytes into tissues emphasising on leukocyte extravasation.

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Unit VII

Molecular neuron biology; General organization of nerve fibers, Axon ultra-structure, Neurotubules and Neurofilaments

Objective: In this unit you will know about general organization of nerve fibers, Axon ultra-structure, Neurotubules and Neurofilaments.

Introduction

Nervous system**,** organized group of cells specialized for the conduction of electrochemical stimuli from sensory receptors through a network to the site at which a response occurs.

All living organisms are able to detect changes within themselves and in their environments. Changes in the external environment include those of light, temperature, sound, motion, and odour, while changes in the internal environment include those in the position of the head and limbs as well as in the internal organs. Once detected, these internal and external changes must be analysed and acted upon in order to survive. As life on Earth evolved and the environment became more complex, the survival of organisms depended upon how well they could respond to changes in their surroundings. One factor necessary for survival was a speedy reaction or response. Since communication from one cell to another by chemical means was too slow to be adequate for survival, a system evolved that allowed for faster reaction. That system was the nervous system, which is based upon the almost instantaneous transmission of electrical impulses from one region of the body to another along specialized nerve cells called neurons.

General organization of nerve fibers

Nervous systems are of two general types, diffuse and centralized. In the diffuse type of system, found in lower invertebrates, there is no brain, and neurons are distributed throughout the organism in a netlike pattern (Fig 1). In the centralized systems of higher invertebrates and vertebrates, a portion of the nervous system has a dominant role in coordinating information and directing responses. This centralization reaches its culmination in vertebrates, which have a well-developed brain and spinal cord. Impulses are carried to and from the brain and spinal cord by nerve fibres that make up the peripheral nervous system.

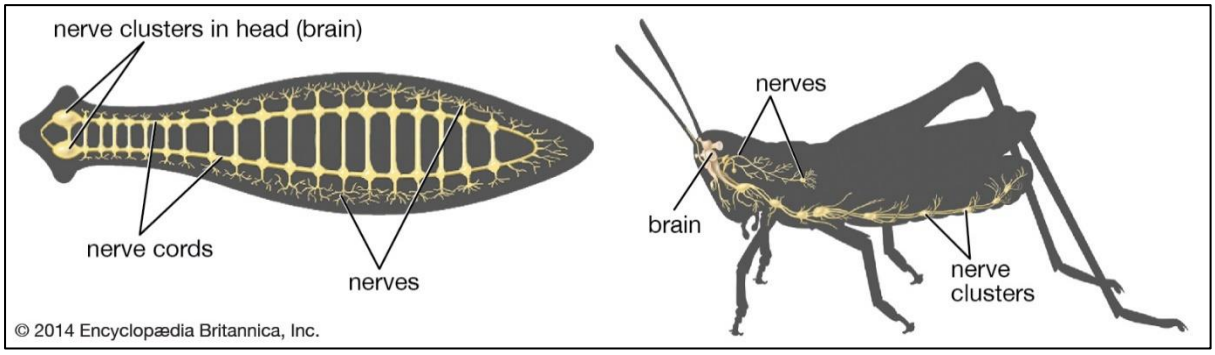

Fig 1: Invertebrate nervous system; nervous system of flat worm (Planaria) and grass hopper

The general features of nervous systems—that is, their function of responding to stimuli and the rather uniform electrochemical processes by which they generate a response. Following that is a discussion of the various types of nervous systems, from the simplest to the most complex.

Nervous systems

The basic pattern of stimulus-response coordination in animals is an organization of receptor, adjustor, and effector units. External stimuli are received by the receptor cells, which, in most cases, are neurons. (In a few instances, a receptor is a non-nervous sensory epithelial cell, such as a hair cell of the inner ear or a taste cell, which stimulates adjacent neurons.) The stimulus is modified, or transduced, into an electrical impulse in the receptor neuron. This incoming excitation, or afferent impulse, then passes along an extension, or axon, of the receptor to an adjustor, called an interneuron. (All neurons are capable of conducting an impulse, which is a brief change in the electrical charge on the cell membrane. Such an impulse can be transmitted, without loss in strength, many times along an axon until the message, or input, reaches another neuron, which in turn is excited.) The interneuron-adjustor selects, interprets, or modifies the input from the receptor and sends an outgoing, or efferent, impulse to an efferent neuron, such as a motor neuron. The efferent neuron, in turn, makes contact with an effector such as a muscle or gland, which produces a response.

In the simplest arrangement, the receptor-adjustor-effector units form a functional group known as the reflex arc. Sensory cells carry afferent impulses to a central interneuron, which makes contact with a motor neuron. The motor neuron carries efferent impulses to the effector, which produces the response. Three types of neurons are involved in this reflex arc, but a two-neuron arc, in which the receptor makes contact directly with the motor neuron, also occurs. In a two-neuron arc, simple reflexes are prompt, short-lived, and automatic and involve only a part of the body. Examples of simple reflexes are the contraction of a muscle in response to stretch, the blink of the eye when the cornea is touched, and salivation at the sight of food. Reflexes of this type are usually involved in maintaining homeostasis.

The differences between simple and complex nervous systems lie not in the basic units but in their arrangement. In higher nervous systems, there are more interneurons concentrated in the central nervous system (brain and spinal cord) that mediate the impulses between afferent and efferent neurons. Sensory impulses from particular receptors travel through specific neuronal pathways to the central nervous system. Within the central nervous system, though, the impulse can travel through multiple pathways formed by numerous neurons. Theoretically, the impulse can be distributed to any of the efferent motor neurons and produce a response in any of the effectors. It is also possible for many kinds of stimuli to produce the same response.

As a result of the integrative action of the interneuron, the behaviour of the organism is more than the simple sum of its reflexes; it is an integrated whole that exhibits coordination between many individual reflexes. Reflexes can occur in a complicated sequence producing elaborate behaviour patterns. Behaviour in such cases is characterized not by inherited, stereotyped responses but by flexibility and adaptability to circumstances. Many automatic, unconditioned reflexes can be modified by or adapted to new stimuli. The experiments of Russian physiologist Ivan Petrovich Pavlov, for example, showed that if an animal salivates at the sight of food while another stimulus, such as the sound of a bell, occurs simultaneously, the sound alone can induce salivation after several trials. This response, known as a conditioned reflex, is a form of learning. The behaviour of the animal is no longer limited by fixed, inherited reflex arcs but can be modified by experience and exposure to an unlimited number of stimuli. The most evolved nervous systems are capable of even higher associative functions such as thinking and memory. The complex manipulation of the signals necessary for these functions depends to a great extent on the number and intricacy of the arrangement of interneurons.

The nerve cell

Nervous system is composed of individual units that are structurally independent of one another and whose internal contents do not come into direct contact. It is now known as the neuron theory, each nerve cell communicates with others through contiguity rather than continuity. That is, communication between adjacent but separate cells must take place across the space and barriers separating them. It has since been proved that Cajal's theory is not universally true, but his central idea—that communication in the nervous system is largely communication between independent nerve cells—has remained an accurate guiding principle for all further study.

There are two basic cell types within the nervous system: neurons and neuroglial cells.

The neuron

In the human brain there are an estimated 85 billion to 200 billion neurons. Each neuron has its own identity, expressed by its interactions with other neurons and by its secretions; each also has its own function, depending on its intrinsic properties and location as well as its inputs from other select groups of neurons, its capacity to integrate those inputs, and its ability to transmit the information to another select group of neurons.

With few exceptions, most neurons consist of three distinct regions, as shown in the diagram (Fig 2): (1) the cell body, or soma; (2) the nerve fibre, or axon; and (3) the receiving processes, or dendrites.

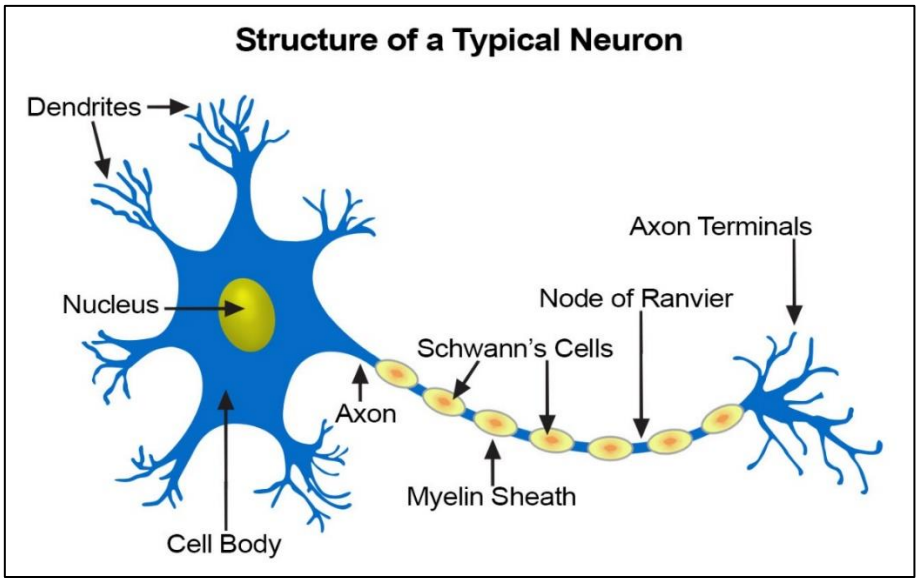

Fig 2: Anatomy of nerve cell

Soma

Plasma membrane

The neuron is bound by a plasma membrane, a structure so thin that its fine detail can be revealed only by high-resolution electron microscopy. About half of the membrane is the lipid bilayer, two sheets of mainly phospholipids with a space between. One end of a phospholipid molecule is hydrophilic, or water attaching, and the other end is hydrophobic, or water repelling. The bilayer structure results when the hydrophilic ends of the phospholipid molecules in each sheet turn toward the watery mediums of both the cell interior and the extracellular environment, while the hydrophobic ends of the molecules turn in toward the space between the sheets. These lipid layers are not rigid

structures; the loosely bonded phospholipid molecules can move laterally across the surfaces of the membrane, and the interior is in a highly liquid state.

Embedded within the lipid bilayer are proteins, which also float in the liquid environment of the membrane. These include glycoproteins containing polysaccharide chains, which function, along with other carbohydrates, as adhesion sites and recognition sites for attachment and chemical interaction with other neurons. The proteins provide another basic and crucial function: those which penetrate the membrane can exist in more than one conformational state, or molecular shape, forming channels that allow ions to pass between the extracellular fluid and the cytoplasm, or internal contents of the cell. In other conformational states, they can block the passage of ions. This action is the fundamental mechanism that determines the excitability and pattern of electrical activity of the neuron.

A complex system of proteinaceous intracellular filaments is linked to the membrane proteins. This cytoskeleton includes thin neurofilaments containing actin, thick neurofilaments similar to myosin, and microtubules composed of tubulin. The filaments are probably involved with movement and translocation of the membrane proteins, while microtubules may anchor the proteins to the cytoplasm.

Nucleus

Each neuron contains a nucleus defining the location of the soma. The nucleus is surrounded by a double membrane, called the nuclear envelope that fuses at intervals to form pores allowing molecular communication with the cytoplasm. Within the nucleus are the chromosomes, the genetic material of the cell, through which the nucleus controls the synthesis of proteins and the growth and differentiation of the cell into its final form. Proteins synthesized in the neuron include enzymes, receptors, hormones, and structural proteins for the cytoskeleton.

Organelles

The endoplasmic reticulum (ER) is a widely spread membrane system within the neuron that is continuous with the nuclear envelope. It consists of series of tubules, flattened sacs called cisternae, and membrane-bound spheres called vesicles. There are two types of ER. The rough endoplasmic reticulum (RER) has rows of knobs called ribosomes on its surface. Ribosomes synthesize proteins that, for the most part, are transported out of the cell. The RER is found only in the soma. The smooth endoplasmic reticulum (SER) consists of a network of tubules in the soma that connects the RER with the Golgi apparatus. The tubules can also enter the axon at its initial segment and extend to the axon terminals.

The Golgi apparatus is a complex of flattened cisternae arranged in closely packed rows. Located close to and around the nucleus, it receives proteins synthesized in the RER and transferred to it via the SER. At the Golgi apparatus, the proteins are attached to carbohydrates. The glycoproteins so formed are packaged into vesicles that leave the complex to be incorporated into the cell membrane.

Axon

The axon arises from the soma at a region called the axon hillock, or initial segment. This is the region where the plasma membrane generates nerve impulses; the axon conducts these impulses away from the soma or dendrites toward other neurons. Large axons acquire an insulating myelin sheath and are known as myelinated, or medullated, fibres. Myelin is composed of 80 percent lipid and 20 percent protein; cholesterol is one of the major lipids, along with variable amounts of cerebrosides and phospholipids. Concentric layers of these lipids separated by thin layers of protein give rise to a high-resistance, low-capacitance electrical insulator interrupted at intervals by gaps called nodes of Ranvier, where the nerve membrane is exposed to the external environment. In the

central nervous system the myelin sheath is formed from glial cells called oligodendrocytes, and in peripheral nerves it is formed from Schwann cells.

While the axon mainly conducts nerve impulses from the soma to the terminal, the terminal itself secretes chemical substances called neurotransmitters. The synthesis of these substances can occur in the terminal itself, but the synthesizing enzymes are formed by ribosomes in the soma and must be transported down the axon to the terminal. This process is known as axoplasmic flow; it occurs in both directions along the axon and may be facilitated by microtubules.

At the terminal of the axon, and sometimes along its length, are specialized structures that form junctions with other neurons and with muscle cells. These junctions are called synapses. Presynaptic terminals, when seen by light microscope, look like small knobs and contain many organelles. The most numerous of these are synaptic vesicles, which, filled with neurotransmitters, are often clumped in areas of the terminal membrane that appear to be thickened. The thickened areas are called presynaptic dense projections, or active zones.

The presynaptic terminal is unmyelinated and is separated from the neuron or muscle cell onto which it impinges by a gap called the synaptic cleft, across which neurotransmitters diffuse when released from the vesicles. In nerve-muscle junctions the synaptic cleft contains a structure called the basal lamina, which holds an enzyme that destroys neurotransmitters and thus regulates the amount that reaches the postsynaptic receptors on the receiving cell. Most knowledge of postsynaptic neurotransmitter receptors comes from studies of the receptor on muscle cells. This receptor, called the end plate, is a glycoprotein composed of five subunits. Other neurotransmitter receptors do not have the same structure, but they are all proteins and probably have subunits with a central channel that is activated by the neurotransmitter.

While the chemically mediated synapse described above forms the majority of synapses in vertebrate nervous systems, there are other types of synapses in vertebrate brains and, in especially great numbers, in invertebrate and fish nervous systems. At these synapses there is no synaptic gap; instead, there are gap junctions, direct channels between neurons that establish a continuity between the cytoplasm of adjacent cells and a structural symmetry between the pre- and postsynaptic sites. Rapid neuronal communication at these junctions is probably electrical in nature.

Dendrites

Besides the axon, neurons have other branches called dendrites that are usually shorter than axons and are unmyelinated. Dendrites are thought to form receiving surfaces for synaptic input from other neurons. In many dendrites these surfaces are provided by specialized structures called dendritic spines, which, by providing discrete regions for the reception of nerve impulses, isolate changes in electrical current from the main dendritic trunk.

The traditional view of dendritic function presumes that only axons conduct nerve impulses and only dendrites receive them, but dendrites can form synapses with dendrites and axons and even somata can receive impulses. Indeed, some neurons have no axon; in these cases nervous transmission is carried out by the dendrites.

The neuroglia

Neurons form a minority of the cells in the nervous system. Exceeding them in number by at least 10 to 1 are neuroglial cells, which exist in the nervous systems of invertebrates as well as vertebrates. Neuroglia can be distinguished from neurons by their lack of axons and by the presence of only one type of process. In addition, they do not form synapses, and they retain the ability to divide throughout their life span. While neurons and neuroglia lie in close apposition to one another,

there are no direct junctional specializations, such as gap junctions, between the two types. Gap junctions do exist between neuroglial cells.

Types of neuroglia

Apart from conventional histological and electron-microscopic techniques, immunologic techniques are used to identify different neuroglial cell types. By staining the cells with antibodies that bind to specific protein constituents of different neuroglia, neurologists have been able to discern two (in some opinions, three) main groups of neuroglia: (1) astrocytes, subdivided into fibrous and protoplasmic types; (2) oligodendrocytes, subdivided into Interfascicular and perineuronal types; and sometimes (3) microglia.

Fibrous astrocytes are prevalent among myelinated nerve fibres in the white matter of the central nervous system. Organelles seen in the somata of neurons are also seen in astrocytes, but they appear to be much sparser. These cells are characterized by the presence of numerous fibrils in their cytoplasm. The main processes exit the cell in a radial direction (hence the name *astrocyte*, meaning "star-shaped cell"), forming expansions and end feet at the surfaces of vascular capillaries.

Unlike fibrous astrocytes, protoplasmic astrocytes occur in the grey matter of the central nervous system. They have fewer fibrils within their cytoplasm, and cytoplasmic organelles are sparse, so that the somata are shaped by surrounding neurons and fibres. The processes of protoplasmic astrocytes also make contact with capillaries.

Oligodendrocytes have few cytoplasmic fibrils but a well-developed Golgi apparatus. They can be distinguished from astrocytes by the greater density of both the cytoplasm and the nucleus, the absence of fibrils and of glycogen in the cytoplasm, and large numbers of microtubules in the processes. Interfascicular oligodendrocytes are aligned in rows between the nerve fibres of the white matter of the central nervous system. In grey matter, perineuronal oligodendrocytes are located in close proximity to the somata of neurons. In the peripheral nervous system, neuroglia that are equivalent to oligodendrocytes are called Schwann cells.

Microglial cells are small cells with dark cytoplasm and a dark nucleus. It is uncertain whether they are merely damaged neuroglial cells or occur as a separate group in living tissue.

Neuroglial functions

The term *neuroglia* means "nerve glue," and these cells were originally thought to be structural supports for neurons. This is still thought to be plausible, but other functions of the neuroglia are now generally accepted. Oligodendrocytes and Schwann cells produce the myelin sheath around neuronal axons. Some constituent of the axonal surface stimulates Schwann cell proliferation; the type of axon determines whether there is loose or tight myelination of the axon. In tight myelination a glial cell wraps itself like a rolled sheet around a length of axon until the fibre is covered by several layers. Between segments of myelin wrapping are exposed sections called nodes of Ranvier, which are important in the transmission of nerve impulses. Myelinated nerve fibres are found only in vertebrates, leading biologists to conclude that they are an adaptation to transmission over relatively long distances.

Another well-defined role of neuroglial cells is the repair of the central nervous system following injury. Astrocytes divide after injury to the nervous system and occupy the spaces left by injured neurons. The role of oligodendrocytes after injury is unclear, but they may proliferate and form myelin sheaths.

When neurons of the peripheral nervous system are severed, they undergo a process of degeneration followed by regeneration; fibres regenerate in such a way that they return to their original target sites. Schwann cells that remain after nerve degeneration apparently determine the route. This route direction is also performed by astrocytes during development of the central nervous system. In the developing cerebral cortex and cerebellum of primates, astrocytes project long processes to certain locations, and neurons migrate along these processes to arrive at their final locations. Thus, neuronal organization is brought about to some extent by the neuroglia.

Astrocytes are also thought to have high-affinity uptake systems for neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA). This function is important in the modulation of synaptic transmission. Uptake systems tend to terminate neurotransmitter action at the synapses and may also act as storage systems for neurotransmitters when they are needed. For instance, when motor nerves are severed, nerve terminals degenerate and their original sites are occupied by Schwann cells. The synthesis of neurotransmitters by neurons apparently also requires the presence of neuroglial cells in the vicinity.

Finally, the environment surrounding neurons in the brain consists of a network of very narrow extracellular clefts. In 1907 Italian biologist Emilio Lugaro suggested that neuroglial cells exchange substances with the extracellular fluid and in this way exert control on the neuronal environment. It has since been shown that glucose, amino acids, and ions - all of which influence neuronal function- are exchanged between the extracellular space and neuroglial cells. After high levels of neuronal activity, for instance, neuroglial cells can take up and spatially buffer potassium ions and thus maintain normal neuronal function.

Classification of Nerve Fibre:

Based on the presence or absence of myelin sheath, they are classified into myelinated or non-myelinated nerve fibers. In the peripheral nervous system, the Schwann cells are responsible for the formation of the myelin sheath.

In the central nervous system, the oligodendroglial cells are responsible for the formation of the myelin sheath. This sheath is broken at regular intervals known as the *Nodes of Ranvier*. Here only the neurilemmal membrane will separate the interior and exterior of the nerve fibre.

Nerve fibers can be classified based on different criteria:

1. Histologically, as myelinated or non-myelinated (Fig 3).

2. Functionally, as afferent (sensory) or efferent (motor).

3. Based on diameter and conduction velocity which is known as Gasser and Erlanger's classification.

4. Based on the type of neurotransmitter released from their terminals as adrenergic, cholinergic, dopaminergic, etc.

In myelinated nerve fibre, the wrapping of the axon by the myelin sheath provided Schwann cell occurs. Whereas in non-myelinated nerve fibre, the Schwann cell just covers the nerve fibre without wrapping. In myelinated nerve fibers, at certain places the myelin sheath is absent. These areas are called as nodes of Ranvier. The protoplasm present in the axon region is known as axoplasm.

The transport of materials within the axoplasm can occur in either direction (from the cell body towards the axon and vice versa). When the substance is transported from the cell body towards the axon terminals, it is known as anterograde transport (fastest—400 mm/day). This type of movement

of axoplasm helps for transport of neurotransmitter, proteins, etc. from the cell body to the end of the nerve terminals.

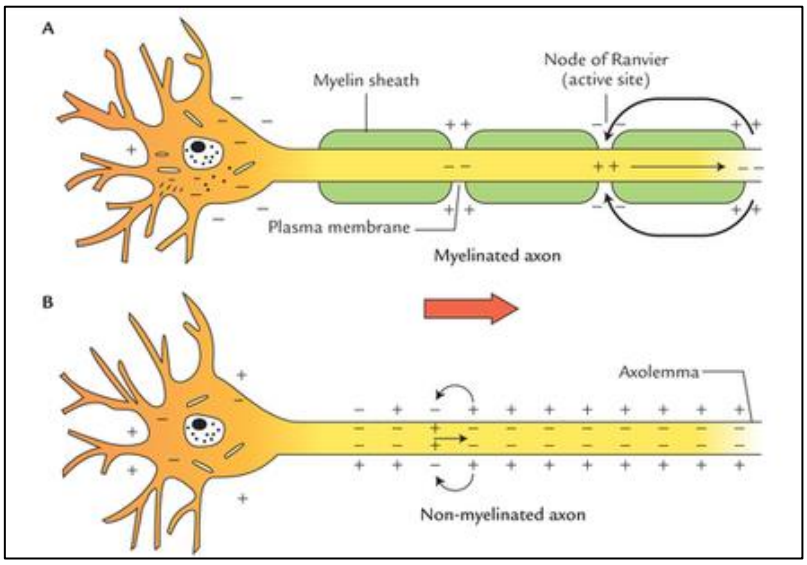

Fig 3: Myelinated and non-myelinated nerve fibre

When the transport of substance occurs in opposite direction (that is from the nerve terminals towards the cell body), it is known as retrograde transport (slowest—200 mm/day). Many of the viruses (rabies virus, polio), bacteria (tetanus), etc. reach the cell bodies in the nervous system because of the retrograde transport.

Myelinogenesis:

Myelinogenesis is the process by which myelination of the nerve fibre takes place. In the peripheral nervous system, the myelinogenesis is contributed by the Schwann cells whereas in the CNS it is being contributed by the oligodendroglial cells. The sheath of Schwann cell wraps the axon by about 80-100 times. The cell membrane lipids form the myelin sheath.

Functions of the Myelin Sheath:

1. In myelinated nerve fibers, the velocity of impulse transmission is faster because the process of depolarization occurs only at the nodes of Ranveir and, therefore, it appears as if the impulses are jumping from one node to the successive node (Fig 4).

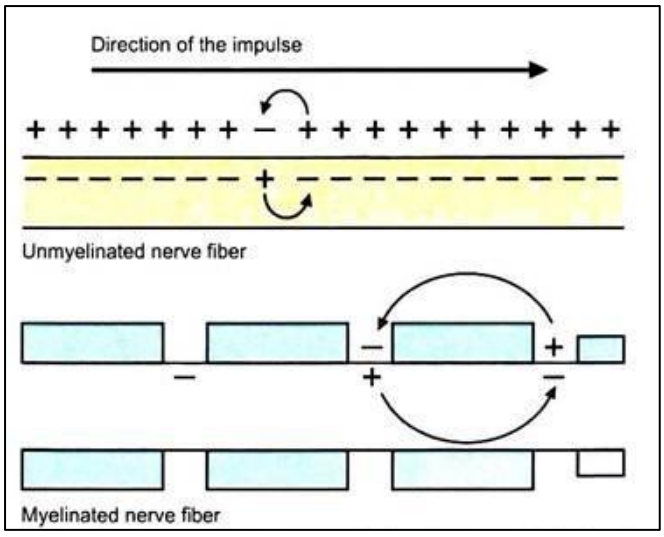

Fig 4: The process of impulse conduction in unmyelinated and myelinated nerve fibres. In myelinated nerve fibre, the impulse jumps from one node of Ranvier to the next

This type of impulse transmission is known as saltatory or leaping type of conduction. Because of this type of impulse transmission, the energy required for conduction is markedly reduced.

2. It acts as a protective sheath minimizing injury to the nerve fibre.

3. It acts as an insulator and prevents cross transmission of impulses from one fibre to the other in a mixed nerve.

Properties of Nerve fibre:

i. Excitability:

When a stimulus is applied, the nerve fibre demonstrates a change in its electrical activity from its resting state.

ii. Conductivity:

It is the ability of the nerve fibre to transmit impulses all along the whole length of axon without any change in the amplitude of the action potential. This type of conduction is termed as decrement less conduction.

iii. Refractory period (Fig 5):

It is the duration after an effective stimulus, when a second stimulus is applied, there will be no response for the second stimulus.

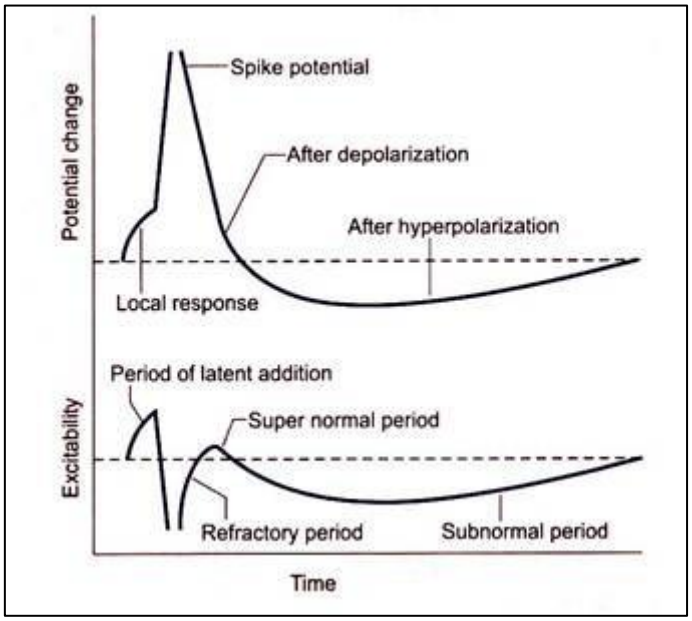

Fig 5: Excitability of the nerve fibre during absolute and relative refractory periods

a. From the time of the application of the stimulus till the initial one-third of the repolarization phase, the nerve fibre excitability will be zero and is completely refractory for the second stimulus. This duration is known as absolute refractory period.

b. Relative refractory period is the duration after an effective stimulus, when a second stimulus, which is slightly above threshold, is applied there will be response for the second stimulus as well.

iv. All or none law:

It states that, when the tissue is stimulated with threshold or more than threshold strength, the amplitude of response will remain the same but for a stimulus of less than threshold strength, there will not be any response.

All or none is obeyed by:

a. A single nerve fibre.

b. A single skeletal muscle fibre.

c. A motor unit.

d. Whole of cardiac muscle.

e. A single fibre of multi-unit smooth muscle.

f. Whole of visceral smooth muscle.

Nerve impulse transmission

Nerve impulse: Nerve impulse is an overall physiological changes that occur in a neuron due mechanical, chemical or electrical disturbance created by a stimulus. It propagation through axon, synapse and neuromuscular junction is called Nerve Impulse conduction.

Nerve impulse transmission along neuron

Transmission of nerve impulse along nerve fibre can be summarized in three steps (Fig 6)

1. Polarization (Resting Potential)
2. Depolarization (Action Potential)
3. Repolarization

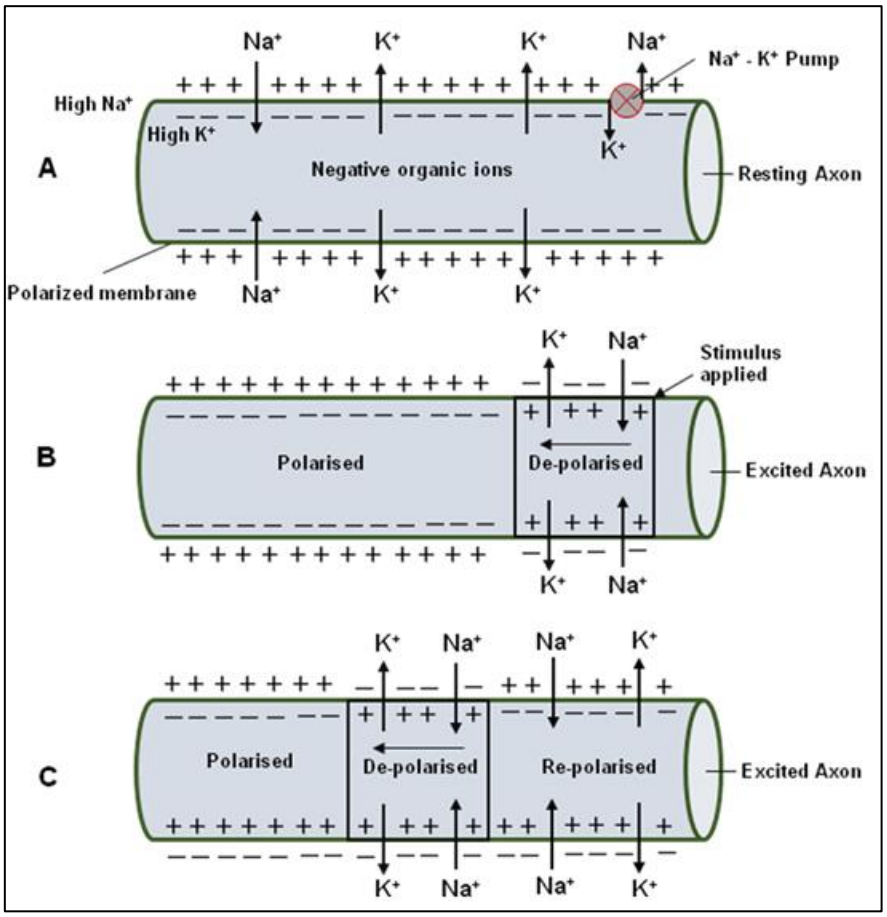

Fig 6: nerve impulse transmission along neuron

Resting potential. The resting potential describes the unstimulated, polarized state of a neuron (at about –70 millivolts).

Action potential:

Unlike a graded potential, an action potential is capable of traveling long distances. If a depolarizing graded potential is sufficiently large, Na^+ channels in the trigger zone open. In response, Na^+ on the outside of the membrane becomes depolarized (as in a graded potential). If the stimulus is strong enough—that is, if it is above a certain threshold level—additional Na^+ gates open, increasing the flow of Na^+ even more, causing an action potential, or complete depolarization (from –70 to about +30 millivolts). This in turn stimulates neighbouring Na^+ gates, farther down the axon, to open. In this manner, the action potential travels down the length of the axon as opened Na^+ gates stimulate neighbouring Na^+ gates to open. The action potential is an all-or-nothing event: When the stimulus fails to produce depolarization that exceeds the threshold value, no action potential results, but when threshold potential is exceeded, complete depolarization occurs.

Polarization (Resting potential):

- A neuron at resting is electrically charged but not conducting.
- The Axoplasm or plasma membrane of a resting neuron is negatively charged as compared to the interstitial fluid.
- The potential difference measured at this stage is called **resting potential** which is about **-70mV**. The interstitial fluid has high concentration of Na+ ion which is about 16 times higher outside the neuron than inside neuron. Similarly, the axoplasm has high concentration of K+ ion which is about 25 times higher inside than in outer interstitial fluids.
- Due to difference in concentration of ions, Na+ ion tends to diffuse into the axoplasm and K+ ion tends to diffuse outside the axoplasm.
- The membrane of neuron at resting is more permeable to K+ ion than Na+ ion. So, K+ leaves the neuron faster than Na+ enter the neuron.
- The difference in permeability results in accumulation of high concentration of cation (+ve charged ion) outside the neuron compared to the concentration of cation inside.
- This state of resting neuron is called **Polarized state** and it is electro-negatively charged.

Depolarization (Action Potential):

- Any stimulus beyond the threshold can initiate an impulse.
- When such stimulus is applied in the resting neuron, it opens the sodium channel. Now the permeability of Na^+ ion suddenly increases at the point of stimulus causing depolarization.
- The diffusion of Na^+ ion increases by 10 times from outside to inside. As a result the axoplasm become positively charges, which is exact opposite to polarized state, so called as **depolarized state** or **reverse polarized state.**
- The depolarization of the membrane stimulates the adjacent voltage channel, so the action potential passes as a wave along the length of neuron.

Repolarization:

- When the concentration of Na+ ion inside axoplasm increases, the permeability to Na+ decreases and the sodium channel starts to close.
- The Na-K pump activates, so that Na+ are pumped out and K+ inside until the original resting potential is restored. The process is known as **repolarization** and it starts from the same point from where depolarization starts.
- The entire process of polarization, depolarization and repolarization occur within fraction of seconds. Now, again the neuron is read for another impulse.

Saltatory conduction:

- Transmission of nerve impulses is very rapid. However, nerve impulse conduction along unmyelinated neuron is slow than that of myelinated neuron. It is because, the myelin sheath act as insulator, so that the impulse have to jump from one *node of Raniver* to another.
- This speed up the conduction process, and this type of conduction is known as **Saltatory conduction.**

Graded potential. A graded potential is a change in the resting potential of the plasma membrane in the response to a stimulus. A graded potential occurs when the stimulus causes Na + or K + gated channels to open. If Na + channels open, positive sodium ions enter, and the membrane depolarizes (becomes more positive). If the stimulus opens K + channels, then positive potassium ions exit across the membrane and the membrane hyperpolarizes (becomes more negative). A graded potential is a local event that does not travel far from its origin. Graded potentials occur in cell bodies and dendrites. Light, heat, mechanical pressure, and chemicals, such as neurotransmitters, are examples of stimuli that may generate a graded potential (depending upon the neuron).

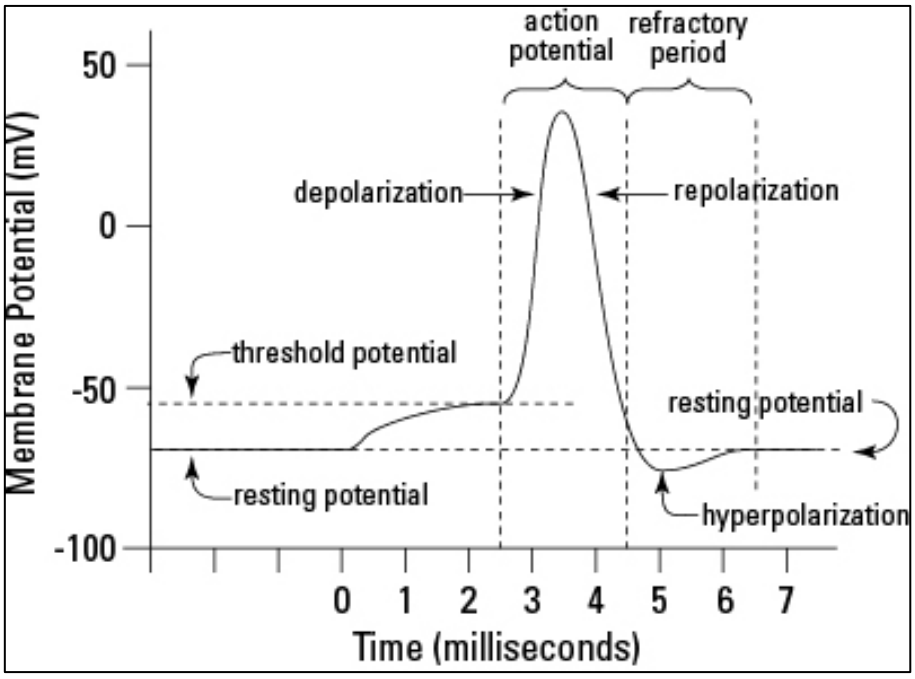

Fig 7: Overall incidence of nerve impulse conduction

The Synapse

The synapse is an area of functional contact between one neuron and another for the purpose of transferring information. Synapses are usually found between the fine terminal branches of the axon of one neuron and the dendrites or cell body of another.

This type of neuron is called axo-dendrite synapse. Sir Charles Sherrington (1861-1954) was the first person who used the term 'synapse' to the junctional points between two neurons.

Types of Synapse

On the basis of transmission of impulses, the synapse is of two types:

i. Electrical synapse and
ii. Chemical synapse. However, generally the term synapse refers to a chemical synapse.

Structure of Synapse

A typical (generalized synapse) consists of a bulbous expansion of a nerve terminal called a pre-synaptic knob lying close to the membrane of a dendrite.

The cytoplasm of the synaptic knob contains mitochondria, smooth endoplasmic reticulum, microfilaments and numerous synaptic vesicles. Each vesicle contains neurotransmitter (chemical substance) responsible for the transmission of the nerve impulse across the synapse. The membrane of the synaptic knob nearest the synapse is thickened and forms the presynaptic membrane. The membrane of the dendrite is also thickened and is called the postsynaptic membrane. These membranes are separated by a gap, the synaptic cleft. The post synaptic membrane contains large protein molecules which act as receptor sites for neurotransmitter and numerous channels and pores.

The two main neurotransmitters in vertebrate nervous system are acetylcholine (ACh) and noradrenaline although other neurotransmitters also exist.

Acetylcholine (ACh) was the first neurotransmitter to be isolated and obtained by Otto Loewi in 1920 from the endings of parasympathetic neurons of the vagus nerve in frog heart. Neurons releasing acetylcholine are described as cholinergic neurons and those releasing noradrenaline are described as adrenergic neurons.

Transmission of Impulses

1. Transmission of Nerve Impulses at an Electrical Synapse

At electrical synapse there is continuity between the presynaptic and postsynaptic neurons. The continuity is provided by the gap junction between the two neurons. The gap junctions are small protein tubular structures that allow free movement of ions between the two neurons. Because of this, the action potential reaching the presynaptic terminal produces potential change in the post-synaptic neuron.

In electrical synapse there is minimal synaptic delay because of the direct flow of electrical current from one neuron into the other through gap junction. Thus impulse transmission across an electrical synapse is always faster than that across a chemical synapse.

At an electrical synapse, the transfer of an impulse occurs by purely electrical means without involving any chemical (neurotransmitter). However, electrical synapses are relatively rare. It is found in the cardiac muscle fibres, smooth muscle fibres of intestine and the epithelial cells of lens. Most impulse transmission across the synapse between neurons takes place at the chemical synapses.

2. Transmission of Nerve Impulse at a Chemical Synapse:

The process of chemical transmission across synapses was discovered by Henry Dale (1936). The physiological importance of synapse for the transmission of nerve impulses was established by McLennan in 1963.

A brief description of the mechanism of synaptic transmission is given below (Fig 8):

(i) When an impulse arrives at a presynaptic knob, calcium ions from the synaptic cleft enter the cytoplasm of the presynaptic knob.

(ii) The calcium ions cause the movement of the synaptic vesicles to the surface of the knob. The synaptic vesicles are fused with the presynaptic membrane and get ruptured (exocytosis) to discharge their contents (neurotransmitter) into the synaptic cleft.

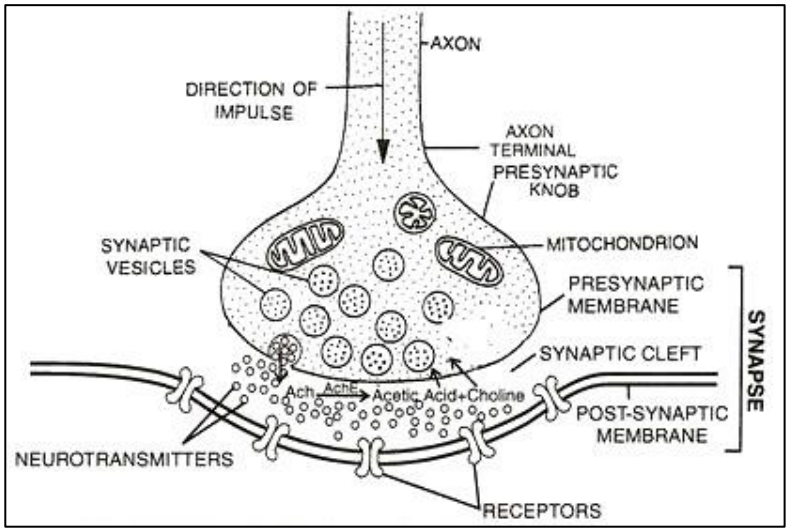

Fig 8: Transmission of nerve impulse at a chemical synapse

(iii) The synaptic vesicles then return to the cytoplasm of the synaptic knob where they are refilled with neurotransmitter.

(iv) The neurotransmitter of the synaptic cleft binds with protein receptor molecules on the post synaptic membrane. This binding action changes the membrane potential of the postsynaptic membrane, opening channels in the membrane and allowing sodium ions to enter the cell.

This causes the depolarization and generation of action potential in the postsynaptic membrane. Thus the impulse is transferred to the next neuron.

(v) Having produced a change in the permeability of the postsynaptic membrane the neurotransmitter is immediately lost from the synaptic cleft. In the case of cholinergic synapses, acetylcholine (ACh) is hydrolysed by an enzyme acetyl cholinesterase (AChE) which is present in high concentration at the synapse.

(vi) The products of the hydrolysis are acetic acid and choline which are reabsorbed into the synaptic knob where they are resynthesized into acetylcholine, using energy from ATP.

Ultra structure of Axon

An **axon**, or nerve fibre, is a long slender projection of a nerve cell, or neuron that conducts electrical impulses away from the neuron's cell body or soma. **Axons** are in effect the primary transmission lines of the nervous system, and as bundles they help make up nerves. Some axons may be quite long,

reaching, for example, from the spinal cord down to a toe. Most axons of vertebrates are enclosed in a myelin sheath, which increases the speed of impulse transmission; some large axons may transmit impulses at speeds up to 90 metres (300 feet) per second.

The chemical constituents of the myelin sheath, giving rise to the polarization optical and X-ray diffraction effects to be described in this section, are the lipoids and the proteins. The lipoids may be classified under three general headings: phosphatids (lecithin, cephalin, and sphingomyelin), cerebrosides (cerebron, kerasin, nervon, and oxynervon), and sterols (chiefly cholesterol and its esters).

In terms of modern protein chemistry little can be said to be known definitely about the protein of the myelin sheath. On the basis of solubility and digestibility data this protein can be classified with the keratins, giving it the name "neurokeratin". As a result of a comparison of the relative amounts of certain constituent amino acids of neurokeratin and the more typical keratins.

(I) Polarized light studies

The myelin sheath of a fresh fibre behaves optically as though it were composed of positive uniaxial elements with optic axes directed radially. This description is in agreement with observations made by viewing the fibres laterally and in cross-sections prepared by the freezing method. With respect to microscopic appearance and optical properties the myelin sheath shows close resemblance to the tubular myelin forms produced by the action of water on the lipoids extractable from nerve, either in a mixture or as single pure components. Usually the protein residue (neurokeratin) takes on the appearance of a mess or network. Under certain conditions there is a marked tendency for the protein to separate out in concentric lamellae or leaflets, a fact readily understandable from the optical analysis, which shows that the protein particles are oriented in a similar manner in the sheaths of fresh fibres.

(II) X-ray diffraction analysis

The patterns obtained from a wide variety of fresh vertebrate myelinated nerves (amphibian and mammalian, peripheral and central) are all essentially alike. They are, moreover, to be ascribed entirely to the myelin sheath (other components, even if present in appreciable quantities, are not ordinarily detected except after drying the nerve). The various diffractions obtained from fresh nerves, considered in the light of the optical evidence cited above, have been interpreted as indicating that the myelin sheath is constructed in the following manner. The basic pattern is one of cylindrical layers wrapped concentrically about the axis cylinder and containing lipoid and protein molecules. Each unit layer has a thickness in a radial direction of approximately 171 Å and is composed of two bimolecular leaflets of lipoid bounded on either side by a layer of protein. The most probable configuration of such a system would be that in which polar groups of the constituent layers are in apposition to form predominantly aqueous phases. Alternating with these are the predominantly hydrocarbon phases containing the paraffin chains of the lipoid molecules. The radial organization has been deduced from the equatorial diffractions obtained from frog sciatic nerves and bull frog motor roots. These consist of spots representing orders of a fundamental spacing of 171 Å. This structure period appears to be unique for nerve, since in general pure lipoids or mixtures of lipoids as found in nerve give spacing to be expected of double layers only. This tendency of the lipoids to form bimolecular layers is commonly observed with long chain compounds of this nature. In the case of nerve it must be supposed that the inclusion of the protein results in the construction of the more complex unit. The particular organization suggested for the nerve sheath rests largely on the facts that the nerve fundamental spacing is more than twice the average lipoid period, and that alternations can be observed in the intensities of the spots assigned to the various orders of the nerve period.

In contrast to the definite, almost crystalline regularity of the sheath structure in the radial direction is the random distribution of the lipoid molecules in the tangential direction, i.e. within the concentric layers. The hydrocarbon chains of the lipoid molecules are distributed in a manner similar to that of liquids. The diffuse meridional diffractions at 4.7 and 9.4 Å. in the nerve pattern correspond to this aspect of the structure. These diffractions are not only of the proper magnitude but their meridional accentuation is also to be expected from this hypothesis. Moreover, the polarized light analysis strongly suggests this lack of well-defined tangential organization, for the single optic axis at any point in the sheath is radially disposed.

Consideration of the semi-fluid consistency of the myelin sheath, as well as the organization outlined above, suggests description of the structure of the sheath (and the similar myelin forms) as being of sematic mixed fluid-crystalline character.

The lipoid molecules must be considered fairly close packed, this does not mean that the hydrocarbon phases of the sheath are necessarily strictly nonporous. Actually the hydrocarbon chains probably occupy less than half the area available in the planes passed tangentially through the predominantly hydrocarbon phases of the concentric layers. Consideration of questions such as these is of importance in connexion with the permeability of the sheath to ions, metabolites, narcotics, etc.

In soft complex structures such as these, conclusions derived from optical and X-ray diffraction studies will represent only an average state of affairs. This makes it difficult to determine the structure with any great degree of precision. This is particularly true with regard to the details of the structure in the neighbourhood of the protein layers. It can be estimated, however, that in every 171 A. period radially in the sheath, approximately 30 A. of thickness are occupied by protein, This would seem to suggest that the protein is present as a single or double layer of particles, or perhaps even as thin sheets. Further investigation of the role of protein in determining sheath ultrastructure is highly desirable.

Neurotubules and Neurofilaments

Adaptation to various shapes (and to carry out coordinated and directed movements) is dependent on complex internal scaffolds of protein filaments and tubules and their associated proteins called the cytoskeleton. The cytoskeletal network extends throughout the cell body, dendrites, and axon. The cytoskeleton is not a fixed structure but undergoes changes during development and growth and after injury.

The cytoskeleton consists of numerous fibrillar organelles called (1) neurotubules (microtubules), each roughly 20-25 nm in diameter, (2) neurofilaments (microfilaments), roughly 10 nm in diameter, and (3) actin microfilaments, about 5 nm in diameter. The tubules and filaments comprise about 25% of the total protein of a neuron. Neurotubules and neurofilaments are found throughout the cytoplasm. As molecular motors, they mediate movement of organelles by transport. The actin microfilaments, primarily located close to the plasma membrane, are critical organelles in growth cones. These tubules and filaments are of variable length with no single element extending the entire length of an axon or dendrite. The tubules are polar structures. Within an axon, the so-called plus end of each tubule is oriented toward the axon terminus and the minus end is oriented toward the cell body. In a dendrite, the polarities of the tubules are mixed, with about half having the plus end oriented toward the cell body and the other half with the minus end oriented toward the cell body. The tubules and filaments consist of a polymer of repeating subunits that are in a dynamic state of flux, continuously growing longer or shorter.

Neurotubules are microtubules found in neurons in nervous tissues. Along with neurofilaments and microfilaments, they form the cytoskeleton of neurons. Neurotubules are undivided hollow cylinders that are made up of tubulin protein polymers and arrays parallel to the plasma membrane in neurons. It has an outer diameter of about 23 nm and an inner diameter, also known as the central core, of about 12 nm. The wall of the neurotubules is about 5 nm in width. There is a non-opaque clear zone surrounding the neurotubule and it is about 40 nm in diameter. Like microtubules, neurotubules are greatly dynamic and the length of them can be adjusted by polymerization and depolymerisation of tubulin.

Despite having similar mechanical properties, neurotubules are distinct from microtubules found in other cell types with regards to their function and intracellular arrangement. Most neurotubules are not anchored in the microtubule organizing centre (MTOC) like conventional microtubules do. Instead, they are released for transport into dendrites and axons after their nucleation in the centrosome. Therefore, both ends of the neurotubules terminates in the cytoplasm instead.

Neurotubules are crucial in various cellular processes in neurons. Together with neurofilaments, they help to maintain the shape of a neuron and provide mechanical support. Neurotubules also aid the transportation of organelles, vesicles containing neurotransmitters, messenger RNA and other intracellular molecules inside a neuron.

Disruption in the integrity and dynamics of neurotubules can interfere with the cellular functions they perform and cause various neurological disorders, like Alzheimer's disease, Lissencephaly.

Neurofilaments are unbranched cylinders formed by actin and other proteins. The neurofilaments proteins, found only in neurons, are members of a family of proteins that include intermediate filament proteins seen in other cells and a protein in glial cells called glial fibrillary acidic protein (GFAP). Neurofilaments (NF) are classed as type IV intermediate filaments found in the cytoplasm of neurons. They are protein polymers measuring 10 nm in diameter and many micrometers in length. Together with microtubules (~25 nm) and microfilaments (7 nm), they form the neuronal cytoskeleton. They are believed to function primarily to provide structural support for axons and to regulate axon diameter, which influences nerve conduction velocity. The proteins that form neurofilaments are members of the intermediate filament protein family, which is divided into six types based on their gene organization and protein structure. Types I and II are the keratins which are expressed in epithelia. Type III contains the proteins vimentin, desmin, peripherin and glial fibrillary acidic protein (GFAP). Type IV consists of the neurofilament proteins L, M, H and internexin. Type V consists of the nuclear lamins, and type VI consists of the protein nestin.

The special properties of these cytoskeletal elements enable them to undergo transitions from stable to dynamic structures. In the fully differentiated neurons the cytoskeleton gives each neuron and its processes (axon and dendrites) (1) mechanical strength and (2) via its neurotubules, the tracks to transport materials between the cell body and its axon terminals. A highly plastic cytoskeleton is exhibited during the development of a neuron or in nerve regeneration following the transection of a peripheral nerve. In these situations, growth cones utilize the cytoskeleton to elongate, retract or rapidly change their shape and to act as mobile sensors.

Function of neurofilaments

Neurofilaments are found in vertebrate neurons in especially high concentrations in axons, where they are all aligned in parallel along the long axis of the axon forming a continuously overlapping array. They have been proposed to function as space-filling structures that increase axonal diameter. Their contribution to axon diameter is determined by the number of neurofilaments in the axon and their

packing density. The number of neurofilaments in the axon is thought to be determined by neurofilament gene expression and axonal transport.

Axonal transport (axoplasmic or axon transport or axoplasmic flow)

The distribution of many substances from the cell body throughout the neuronal processes and from the processes to the cell body is carried out by axonal transport. It is called anterograde or orthograde axonal transport when the direction of movement is away from the cell body into the dendrites and axon and retrograde axonal transport when the direction is from the dendrites and axon toward the cell body. Transport comprises two general rates, which occur in all dendrites and axons: (1) a fast rate with movement of 200-400 mm per day and (2) a slow rate of about 1-5 mm

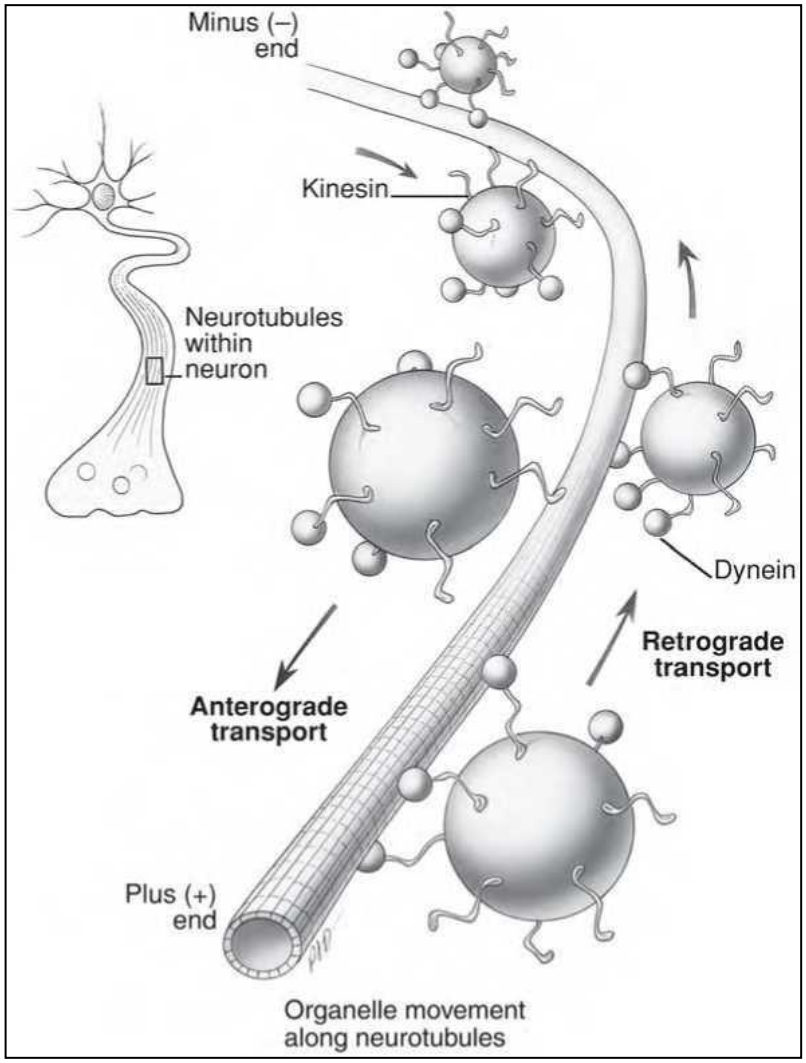

Fig 9: Schematic illustrating the anterograde and retrograde transport of synaptic vesicles and other organelles along neurotubules. After their components are synthesized within the endoplasmic reticulum and GA, the organelles are assembled within the cell body. While in the cell body, the organelles bind with the motor proteins kinesin and dynein. Kinesin has the means to power their rapid movement via fast axonal anterograde transport to the plus (+) end of each neurotubule toward the nerve terminals. The motor is presumed to be transported back to the cell body in an inactive form. The organelle-bound kinesin molecules interact transiently with the microtubule during the anterograde transport via the neurotubule. The retrograde motor protein dynein is transported to the terminal in an inactive form, becomes activated, binds to degraded membranes and organelles, and

then is conveyed by retrograde transport to the minus (-) end of the microtubules toward the cell body for disposal. Kinesin appears to have a fan-shaped tail that binds to the organelle to be moved and two globular heads that bind to the neurotubule. A hingelike site is present midway along the kinesin molecule. The similarities between kinesin and myosin of muscle suggest that the movement is produced by the sliding of kinesin molecules along the tracks of the neurotubules. Neurons have adapted an ancient mechanism of transport, in that kinesin and dynein are present in single-celled organisms and eukaryotic cells.

Per day. There is both anterograde and retrograde fast transport, but only anterograde slow transport. The anterograde fast transport conveys mitochondria and the precursors of smooth endoplasmic reticulum, synaptic vesicles, and plasma membrane. The fast retrograde system includes conveying of such structures as mitochondria, "multivesiculate" bodies (can be degradative structures), and vesicles containing such ligands as nerve growth factors (see "Neurotropic Factors and Tropic Factors") taken up by receptor-mediated endocytosis. Mitochondria travel in both, or either, the anterograde and retrograde direction. The neurotubules in association with certain force-generating motor proteins (neurotubule-based motors) act as intracellular molecular engines for fast transport, also called neurotubule-dependent transport. This fast transport is generated by the molecular motor proteins kinesin and dynein that are linked with ATP (Fig 9). They are responsible for generating the forces for the organelle movements that underlie neurotubular axonal transport. ATP is obligatory, as it furnishes the energy for the fast transport in either direction. These proteins and ATP seem to provide a mechanistic basis for microtubules-associated movement.

The following is a current scheme describing the role of these molecular motor (motility) proteins in fast transport (Fig 9). Both anterograde motor kinesin and retrograde dynein attach to appropriate binding sites on the precursors or organelle to be transported. The organelle is conveyed by anterograde transport along the "rail" of the track on the neurotubule by the "molecular motors" of kinesin and is powered by ATP on the organelle toward the plus (+) or axon terminal end of the neurotubule. During this phase, the dynein is transported in an inactive form. The kinesin is then transported back in an inactive form to the cell body for recycling. The retrograde motor dynein, after being transported in an inactive form to the terminal, is activated and becomes the motor to organelles that are destined to be transported along the rails of the track on the neurotubules toward their minus (-) or cell body ends. Each neurotubule contains several tracks along which different particles move. On a single neurotubule, (1) a vesicle can pass another vesicle moving in the same direction on a separate track or (2) two vesicles can move bi-directionally in opposite directions simultaneously on separate tracks. In addition, a vesicle can shift from one to another tubule.

The slow axonal transport is anterograde and involved with the movement of soluble enzymes and the components of the cytoskele-ton and plasma membrane. Proteins and other substances are conveyed to renew and maintain the axoplasm of mature neurons and to supply the axoplasm for axon and dendrite growth of developing and regenerating neurons. The protein dynamin has been suggested to be the motor protein with a role in slow transport.

Conceptually, axonal transport is an expression of the unity of the neuron in that, through transport, a continuous communication is maintained between the cell body and its processes. By this means, the cell body is kept informed of the metabolic needs and condition of its most distal parts. Through axonal uptake of extracellular substances, such as nerve growth factor followed by retrograde transport, the cell body can sample the extracellular environment. However, retrograde transport has its debit side, in that through this mechanism, neurotropic viruses such as rabies, herpes simplex, and poliomyelitis are conveyed to the central nervous system. Defects in microtubules might be involved in some human neurologic disorders.

Probable questions:

1. What do you mean by diffused nervous system?
2. What do you mean by centralised nervous system?
3. Define reflex arc.
4. What is neuron theory?
5. Give detail structure of neuron with suitable diagram.
6. What is cell body?
7. What is neuroglia?
8. Discuss the types of neuroglia?
9. Distinguish sensory and motor neuron.
10. Discuss with diagram about myelinated nerve fibre.
11. Elaborate the functions of the myelin sheath.
12. Discuss the properties of nerve fibre.
13. Discuss transmission of nerve impulse at a chemical synapse.
14. What is refractory period?
15. Elaborate all or none law.
16. Discuss the nerve impulse transmission along neuron with proper diagram.
17. Discuss the ultra-structure of axon.
18. Write short notes on neurotubules.
19. Write short notes on neurofilaments.
20. Describe axonal transport with diagram.

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Unit VIII

Neurosecretory cell: Occurrence, staining behaviour, neurosecretion in invertebrates

Objective: In this unit you will know about neurosecretory cell; its occurrence, staining behavior and neurosecretion in invertebrates.

Introduction

Neurosecretory cell, a type of neuron, or nerve cell, whose function is to translate neural signals into chemical stimuli. Such cells produce secretions called neurohormones that travel along the neuron axon and are typically released into the bloodstream at neurohemal organs, regions in which the axon endings are in close contact with blood capillaries.

Being a double specialized cell, the neurosecretory cell exhibits the fundamental features of a neuron including bioelectrical activity. It receives afferent connections by way of synaptic junctions, both cholinergic and monoaminergic, and gives responses in the shape of action potentials. It shows also the character of a glandular cell which secretes granules of varying shape originating from the Golgi apparatus. The granules are driven along the processes, mainly the axon, down to their endings, and bear hormonal substances. The hormones are released either in the vicinity of vessels in neurohemal storage organs, or in contact with glandular cells (neurosecretory innervation).

The synthesis of secreted material might partly occur progressively along the axon. It appears likely that hormones might be released not only at the ending, but also along the course of the axon. The nerve impulse conducted by the neurosecretory neuron itself is likely to be responsible for hormone release. The morphological and biochemical aspects of the mechanism of hormone release, for instance the part possibly played by acetylcholine, remain uncertain. There seems to be a conspicuous depletion of the dense content of granules. Attention is drawn to two different kinds of neurosecretory fibers and granules respectively bearing peptidic hormones or other substances which might be monoamines in some neurosecretory systems. The comparison between the monoaminergic and neurosecretory neurons leads to the necessity of a physiological definition of a neurosecretory cell: the product of a neurosecretory cell is of a hormonal nature.

Neurosecretory cells are present in most multicellular animals. They are present in clusters in the medial and lateral parts of the brain, control corpora allata activity by producing juvenile hormone during the larval or nymphal instars, the phase between periods of molting in insects. They are usually distinguished from other neurons by the unusually large size of the cell nucleus, axon endings, and the cell itself.

The neurosecretory cell as a nerve cell

The morphology of these cells is fairly similar to that of ordinary neurons. They are unipolar in lower and multipolar in higher Vertebrates. Attention has been paid to dendrites only when these appeared to be loaded with stainable neurosecretory material and to have a particular destination such as terminating in the ependymal lining.

The cytological characteristics of neurosecretory cells are also, on the whole, those of other neurons: a large vesicular nucleus with broken-up chromatin containing, generally, a single nucleolus, Nissl-bodies, situated as a rule in a peripheral position, neurofibrils, small-sized mitochondria, and finally, 'at the ultrastructural level, neurofilaments and neurotubules.

The neurosecretory cell as a glandular cell

The glandular characteristics of neurosecretory cells are apparent from observations of the cells during spontaneous or experimentally induced variations in activity. The nucleus is frequently described as having an irregular outline, sometimes showing deep invaginations of the nuclear membrane resulting in a particularly distorted shape. Increased nucleolar size is often considered a good criterion for active secretion. Golgi structures indeed do play a part in the formation of neurosecretory material. Ribosomes are always present in a great number, either along ergastoplasmic membranes at the cell periphery, or non-bound as isolated ribosomes and polysomal formations. Consequent on ribosomal abundance, clear or dark cellular aspects result which are related to cell maturation or functional activity. The relation between Nissl-substance and production of neurosecretory material is considered to be a more indirect one reflecting the general role of ergastoplasm in protein synthesis.

Enzymatic activities of neurosecretory cells

Numerous enzymes are present in the vertebrate neurosecretory cells. A few of them only will he discussed here in relation to their possible functional importance for the neurosecretory process. Phosphatases, especially acid phosphatases, are present in neurosecretory cells and have been frequently used as a significant test of secretory activity. The hypothalamic neurosecretory neurons as non-cholinergic. In the neurophysiological sense this seems more and more likely, though it does not rule e out the possibility of acetylcholine production by these neurons and the presence of this transmitter in the neurosecretory axon endings.

Simplified Aldehyde-Fuchsin Staining of Neurosecretory Cells

Gomori's original aldehyde-fuchsin method has been modified by the combination of Halmi's counter stain with Gabe's preparation, consisting of basic fuchsin, 1 gm; boiling water, 200 ml; with HC1, 2 ml and paraldehyde, 2 ml added after cooling and filtering. The solution so made was allowed to ripen 3-4 days at room temperature, and the precipitate which formed was filtered off and dried at 55-60°C. The staining solution consisted of 0.5 gm of the dry precipitate dissolved in 100 ml of 70% alcohol. The staining follows original procedures except that it is very important to bring slides from water to 70% alcohol before placing them in the aldehyde-fuchsin solution and also to remove all excess staining solution by rinsing in 95% alcohol after staining. The staining solution is stable for at least 6 months.

Prepare aldehyde-fuchsin by the method of Gabe; that is, to 200 ml of boiling water, add 1 gm of basic fuchsin (C.I.677), boil 1 min, cool, filter. To the filtrate add 2 ml each of concentrated HCI and paraldehyde. Leave stoppered at room temperature. Each day after preparation of this solution withdraw a drop and place it on a filter paper. The fuchsin will be found to decrease and the precipitate to increase in quantity. When the solution has lost its reddish fuchsin colour (34 days), filter it, and discard the filtrate. Dry the precipitate on the filter paper (the paraffin oven at its usual temperature is suitable for this). The dry crystals can then be removed from the filter paper and stored in a reagent bottle. The yield is about 1.9 gm.

To make the staining solution, dissolve 0.25 gm of dry stain in 50 ml of 70% alcohol. This solution will keep for at least 6 months with no apparent change in behaviour.

Staining procedure

1. Deparaffinize and hydrate sections in the usual way.

2. Oxidize in Gomori's fluid (1941) for 1 min (0.15 gm of $KMnO_4$ in 50 ml of water containing 0.1 ml of concentrated H_2SO_4).

3. Rinse in SO_3 solution (2.5% sodium bisulphite) until all permanganate stain is removed (a few seconds).

4. Rinse in distilled water.

5. Transfer through 30% to 70% alcohol.

6. Stain in the aldehyde-fuchsin solution for 2-10 min.

7. Quickly wipe the back of the slide; and rinse in 95% alcohol.

8. Transfer the slide to a second bath of 95% alcohol for 2-5 min (until no more aldehyde-fuchsin comes away).

9. Bring the water through 70% and 30% alcohols.

10. Counterstain in Halmi's mixture for 20-30 sec.

11. Wipe the back of the slide and differentiate in 95% alcohol containing 0.2% acetic acid until no more stain comes away (2-3 min).

12. Rinse in 95% alcohol.

13. Bring through 2 washes of absolute alcohol to xylene, and mount in balsam. Or,

13a. Bring through 2 washes of absolute alcohol and mount in Euparal.

This method is most satisfactory for staining invertebrate neurosecretions. Results are uniform over long periods because the aldehyde-fuchsin does not deteriorate rapidly, and the use of alcohol rinsing avoids precipitation of any excess stain.

Neurosecretion

Neurosecretion is generally understood to mean release of peptides or amines from specialized neurons into the circulation. In mammals, the classical neurosecretory systems secrete oxytocin or vasopressin from axon terminals in the posterior pituitary and also peptides and amines controlling the anterior pituitary from terminals in the median eminence. Invertebrates have different systems not considered here, but with similar function.

Neurosecretion is the storage, synthesis and release of hormones from neurons. These neurohormones, produced by neurosecretory cells, are normally secreted from nerve cells in the brain that then circulate into the blood. These neurohormones are similar to nonneural endocrine cells and glands in that they also regulate both endocrine and nonendocrine cells. Neurosecretion cells also release their product farther than normal neurons, which only secrete short distances, into the extracellular space some distance from the target cell.

Discovery

In 1928, Ernst Scharrer hypothesized that neurosecretory neurons in the hypothalamus of teleost fish, Phoxinus laevis, had secretory activity similar to that of endocrine gland cells. As more became known about neurosecretory cells, the difference between the actions of nerve communication and endocrine hormone release become less clear. Like the average neuron, these cells conduct electrical impulses along the axon but unlike these neurons, neurosecretion produces neurohormones that are released into the body's circulation. Combining the properties of the nervous and endocrine, these cells have the capacity to affect nerves through chemical messengers. Neurosecretion is a broad area of study and must be further observed to be better understood.

Neurosecretion plays a major role in the life of invertebrates. The neurosecretory cell has been harnessed to direct a variety of physiological functions.

Neurosecretion in insects

The neurosecretory system in insects composed of numerous sets of neurosecretory cells situated in the brain and ventral nerve cord. In insects, the neuroendocrine complex comprises the neurosecretory system stomatogastric nervous system. The neuroendocrine complex constitute, neurosecretory cells (NSC) of brain, a pair of corpora cardiaca (CC), a pair of corpora allata (CA), the frontal ganglion (FG) and the hypocerebral ganglion (HG).

The corpora cardiaca as the nervous structures attached with the brain on the ventral side. In most of the insects, the corpora cardiaca was closely associated with paired glandular organs the corpora allata, where as in higher Diptera corpus cardiacum is incorporated with the corpus allatum in to the ring gland encircling the aorta. In Ephemeroptera and Odonata the corpora cardiaca are simple structures whereas the nervous and glandular elements are undifferentiated from one another. In Dictyoptera the glandular lobe primarily embodies the secretary cells. In certain coleopteran, *Hydrophilus olivaceus* the body of corpora cardiaca was differentiated into medulla and cortex. In mosquitoes the corpora cardiaca are very simple and secretary cells are not observed.

Corpora cardiaca are a pair of neuroglandular bodies that found at the back of the brain and on both sides of the aorta. The corpora cardiaca has two parts which are well separated in locusts. These are intrinsic secretory cells and terminating extrinsic axons are situated at the surface of the anterior secretory lobe functioning as a neurohaemal organ. During the various physiological processes in insects especially at the time of larval development, pupal diapause or in sexual maturity, mating, the corpora cardiaca shows variation in the volume.

The corpora allata are small, a pair of juvenile hormone producing glands. The corpora allata attached to the brain by a nerve that also passes through the corpora cardiaca. They are compact organs of tightly packed cells surrounded by a tough membranous sheath. They secrete the juvenile hormone, which regulate growth and reproduction (Gullan & Cranston, 2005; Triplehorn & Johnson, 2005). In mature reproductive forms of insects, the corpora allata plays an important role in ovarian development `and sexual maturation.

Insects play a large role in what is known about neurosecretion. In simpler organisms neurosecretion mechanisms regulate the heart, the process of metamorphosis, and directly influences the development of the gonadal function. In more advanced organisms the gonadal function is manipulated by the intermediary endocrine processes. Axons from neurosecretory cells trace to corpora cardiaca and corpora allata and produce and secrete a brain hormone which insect

physiologists suspect is bound to a large carrier protein. Although the function is unknown, there are a multitude of these cells found in the ventral ganglia of the nerve cord. Neurosecretory cells, found in clusters in the medial and lateral parts of the brain, control corpora allata activity by producing juvenile hormone during the larval or nymphal instars, the phase between periods of molting in insects. The production of this hormone inhibits the insect during the conversion to maturity and reactivating once the fully-grown adult is prepared for reproduction. The 3rd International Symposium on Neurosecretion at the University of Bristol discussed the intracellular structure of the neurosecretory cells and the migration path to the target organs or vascular fluid areas by neurosecretory granules. More is being discovered on the identification of granules in hormones and the linking of their development with the organism's physiologic state.

Probable questions:

1. What is neurosecretory cell?
2. What do you mean by neurohormone?
3. Write short notes on neuro secretory cells.
4. Describe Aldehyde-Fuchsin staining of neurosecretory cells.
5. Discuss neurosecretion in insect.
6. Write short note on juvenile hormone.

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The study materials of this book have been collected from various books, e-books, journals and other e sources.

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Director
Prof. Manas Mohan Adhikary
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE THEORY PAPER (ZET -404)

CYTOGENETICS AND MOLECULAR BIOLOGY

Unit I -Developmental Genetics and Genomics

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 404 (CYTOGENETICS AND MOLECULAR BIOLOGY)	I	Genetic regulation during development: Gradients in early embryogenesis in Drosophila. Cell fate & signalling pathways. Gap genes; segment polarity genes; axis formation; homeotic genes; homeo-domains; Hox genes & HOM-c genes.	1.0	1	1	
	II	Structural genomics: High resolution chromosome map-RFLP RAPD, fluorescence in situ hybridization; radiation hybrid mapping ; physical mapping of genomes ; genome sequencing		1	1	
	III	Functional genomics: Study of gene interaction by the yeast two hybrid system; study of developmental regulation by using DNA-chips		1	1	
	IV	Comparative genomics: Orthologuos, Paraloguos and Gene displacement, Phylogenetic finger printing.		1	1	

Unit-I

Genetic regulation during development: Gradients in early embryogenesis in Drosophila. Cell fate & signalling pathways. Gap genes; segment polarity genes; axis formation; homeotic genes; homeo- domains; Hox genes & HOM-c genes

Objective: In this unit we will discuss about genetic control on development of Drosophila melanogaster. Different gene and their role in attern and organ formation will e discussed including Homeo Box genes.

Introduction:

It is the fly *Drosophila melanogaster* (Figure 21-23), more than any other organism, that has transformed our understanding of how genes govern the patterning of the body. The anatomy of *Drosophila* is more complex than that of *C. elegans*, with more than 100 times as many cells, and it shows more obvious parallels with our own body structure. Surprisingly, the fly has fewer genes than the worm—about 14,000 as compared with 19,000. On the other hand, it has almost twice as much noncoding DNA per gene (about 10,000 nucleotides on average, as compared with about 5000). The molecular construction kit has fewer types of parts, but the assembly instructions—as specified by the regulatory sequences in the non-coding DNA—seem to be more voluminous.

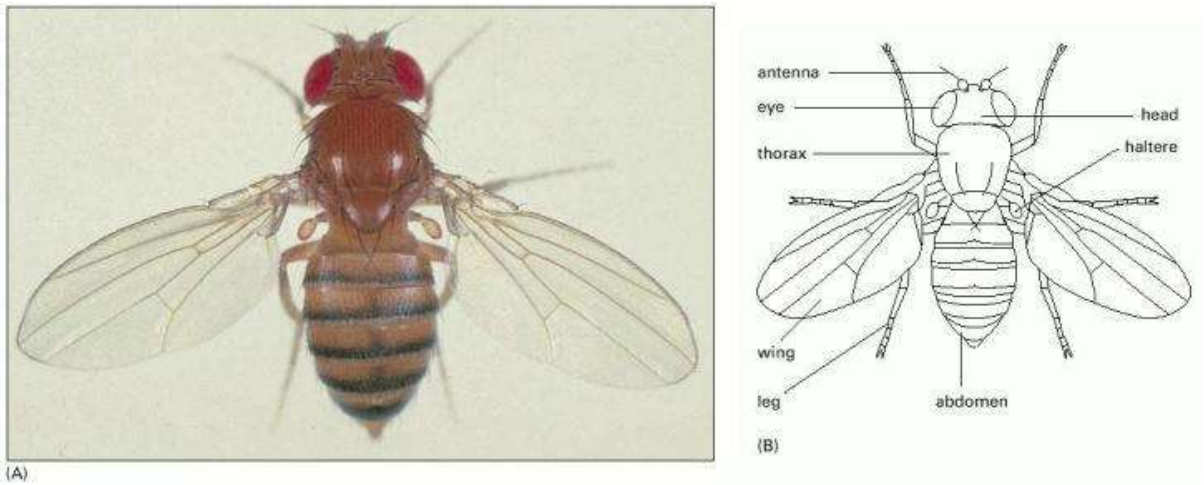


Figure 21-23 : Dorsal view of a normal adult fly. (A) Photograph. (B) Labelled drawing.

Decades of genetic study, culminating in massive systematic genetic screens, have yielded a catalogue of the developmental control genes that define the spatial pattern of cell types and body structures of the fly, and molecular biology has given us the tools to watch these genes in action. By *in situ* hybridization using DNA or RNA probes on whole embryos, or by staining with labelled antibodies to reveal the distribution of specific proteins, one can observe directly how the internal states of the cells are defined by the sets of regulatory genes that they express at different times of development. Moreover, by analysing animals that are a patchwork of mutant and nonmutant cells, one can discover how each gene operates as part of a system to specify the organization of the body.

Most of the genes controlling the pattern of the body in *Drosophila* turn out to have close counterparts in higher animals, including ourselves. In fact, many of the basic devices for defining the body plan and patterning individual organs and tissues are astonishingly similar. Thus, quite surprisingly, the fly has provided the key to understanding the molecular genetics of our own development.

Flies, like nematode worms, are ideal for genetic studies: cheap to breed, easy to mutagenize, and rapid in their reproductive cycle. But there is a more fundamental reason why they have been so important for developmental geneticists. As emphasized earlier, as a result of gene duplications, vertebrate genomes often contain two or three homologous genes corresponding to a single gene in the fly. A mutation that disrupts one of these genes very often fails to reveal the gene's core function, because the other homologs share this function and remain active. In the fly, with its more economical gene set, this phenomenon of genetic redundancy is less prevalent. The phenotype of a single mutation in the fly therefore more often directly uncovers the function of the mutant gene.

The Insect Body Is Constructed as a Series of Segmental Units

The timetable of *Drosophila* development, from egg to adult, is summarized in Figure 21-24. The period of *embryonic development* begins at fertilization and takes about a day, at the end of which the embryo hatches out of the egg shell to become a *larva*. The larva then passes through three stages, or *instars*, separated by moults in which it sheds its old coat of cuticle and lays down a larger one. At the end of the third instar it pupates. Inside the *pupa*, a radical remodelling of the body takes place—a process called *metamorphosis*. Eventually, about nine days after fertilization, an adult fly, or *imago*, emerges.

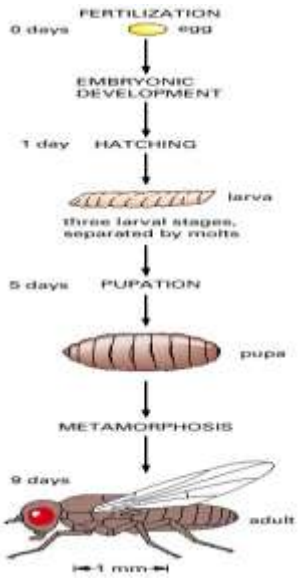


Figure 21-24: Synopsis of *Drosophila* development from egg to adult fly

The fly consists of a head, with mouth, eyes, and antennae, followed by three thoracic segments (numbered T1 to T3), and eight or nine abdominal segments (numbered A1 to A9). Each

segment, although different from the others, is built according to a similar plan. Segment T1, for example, carries a pair of legs, T2 carries a pair of legs plus a pair of wings, and T3 carries a pair of legs plus a pair of halteres—small knob-shaped balancers important in flight, evolved from the second pair of wings that more primitive insects possess. The quasi-repetitive segmentation develops in the early embryo during the first few hours after fertilization (Figure 21-25), but it is more obvious in the larva (Figure 21-26), where the segments look more similar than in the adult. In the embryo it can be seen that the rudiments of the head, or at least the future adult mouth parts, are likewise segmental. At the two ends of the animal, however, there are highly specialized terminal structures that are not segmentally derived.

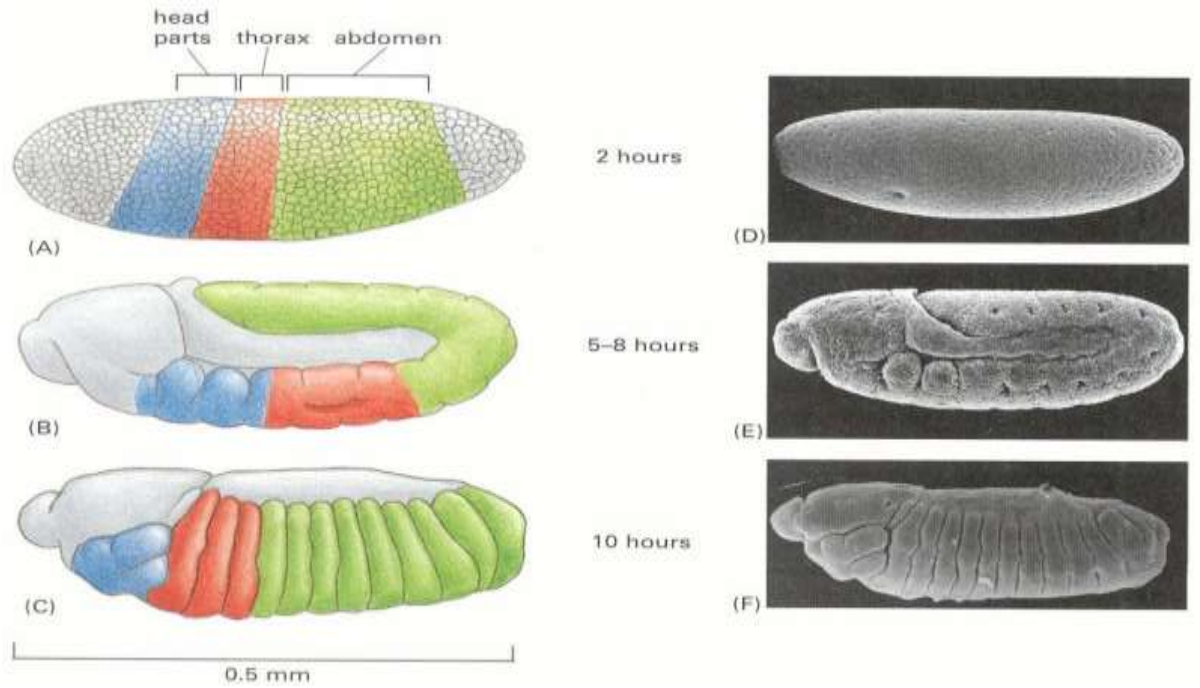


Figure 21-25 : **The origins of the *Drosophila* body segments during embryonic development. The embryos are seen in side view in drawings (A-C) and corresponding scanning electron micrographs (D-F). (A and D) At 2 hours the embryo is at the *syncytial blastoderm* stage (see Figure 21-51) and no segmentation is visible, although a fate map can be drawn showing the future segmented regions (*color* in A). (B and E) At 5–8 hours the embryo is at the *extended germ band* stage: gastrulation has occurred, segmentation has begun to be visible, and the segmented axis of the body has lengthened, curving back on itself at the tail end so as to fit into the egg shell. (C and F) At 10 hours the body axis has contracted and become straight again, and all the segments are clearly defined. The head structures, visible externally at this stage, will subsequently become tucked into the interior of the larva, to emerge again only when the larva goes through pupation to become an adult.**

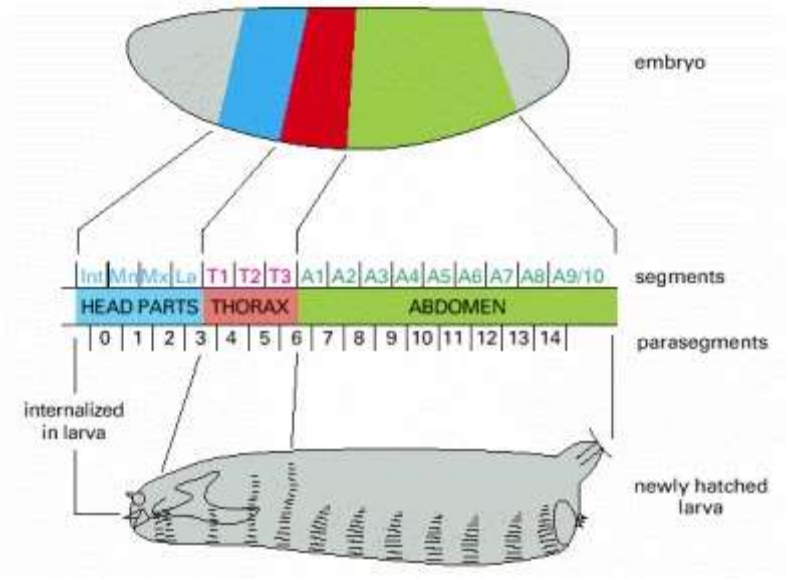


Figure 21-26 The segments of the *Drosophila* larva and their correspondence with regions of the blastoderm. The parts of the embryo that become organized into segments are shown in color. The two ends of the embryo, shaded *gray*, are not segmented and become tucked into the interior of the body to form the internal structures of the head and gut. (The future external, segmental structures of the adult head are also transiently tucked into the interior in the larva.) Segmentation in *Drosophila* can be described in terms of either segments or parasegments: the relationship is shown in the middle part of the figure. Parasegments often correspond more simply to patterns of gene expression. The exact number of abdominal segments is debatable: eight are clearly defined, and a ninth is present vestigially in the larva, but absent in the adult. The boundaries between segments are traditionally defined by visible anatomical markers; but in discussing gene expression patterns it is often convenient to draw a different set of segmental boundaries, defining a series of segmental units called *parasegments*, half a segment out of register with the traditional divisions (see Figure 21-26).

Drosophila Begins Its Development as a Syncytium

The egg of *Drosophila* is about 0.5 mm long and 0.15 mm in diameter, with a clearly defined polarity. Like the eggs of other insects, but unlike vertebrates, it begins its development in an unusual way: a series of nuclear divisions without cell division creates a syncytium. The early nuclear divisions are synchronous and extremely rapid, occurring about every 8 minutes. The first nine divisions generate a cloud of nuclei, most of which migrate from the middle of the egg toward the surface, where they form a monolayer called the *syncytial blastoderm*. After another four rounds of nuclear division, plasma membranes grow inward from the egg surface to enclose each nucleus, thereby converting the syncytial blastoderm into a *cellular blastoderm* consisting of about 6000 separate cells (Figure 21-27). About 15 of the nuclei populating the extreme posterior end of the egg are segregated into cells a few cycles earlier;

these *pole cells* are the germ-line precursors (primordial germ cells) that will give rise to eggs or sperm.

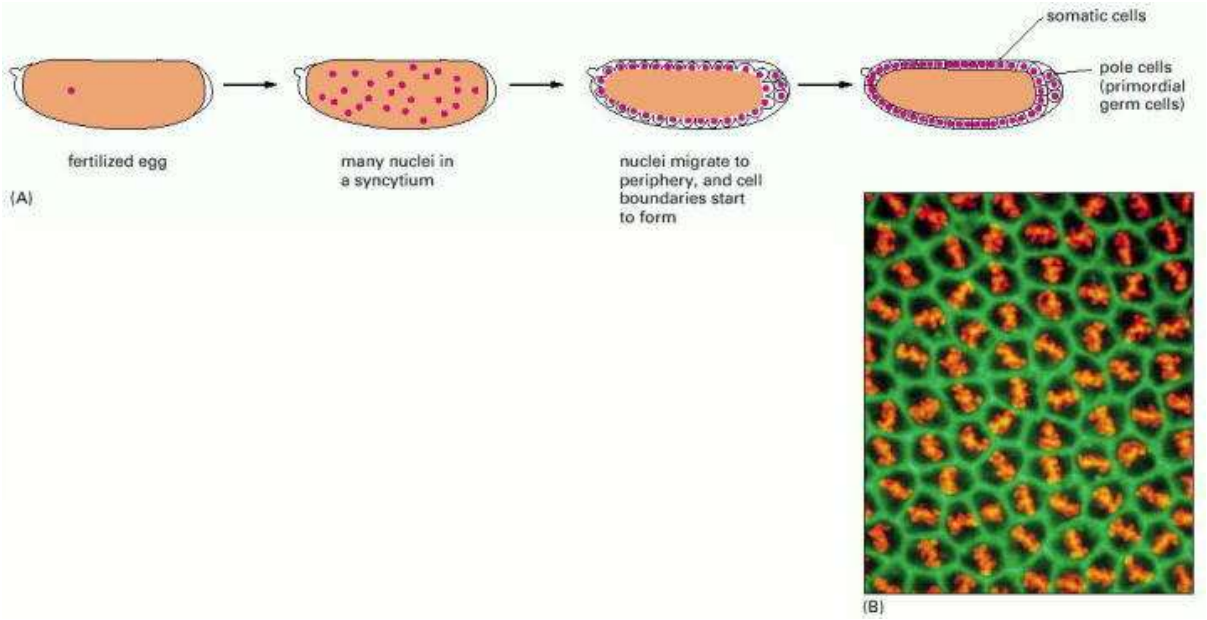


Figure 21-27 Development of the *Drosophila* egg from fertilization to the cellular blastoderm stage.(A) Schematic drawings. (B) Surface view—an optical-section photograph of blastoderm nuclei undergoing mitosis at the transition from the syncytial to the cellular blastoderm stage. Actin is stained *green,* chromosomes *orange.*

Up to the cellular blastoderm stage, development depends largely—although not exclusively—on stocks of maternal mRNA and protein that accumulated in the egg before fertilization. The frantic rate of DNA replication and nuclear division evidently gives little opportunity for transcription. After cellularization, cell division continues in a more conventional way, asynchronously and at a slower rate, and the rate of transcription increases dramatically. Gastrulation begins a little while before cellularization is complete, when parts of the sheet of cells forming the exterior of the embryo start to tuck into the interior to form the gut, the musculature, and associated internal tissues. A little later and in another region of the embryo, a separate set of cells move from the surface epithelium into the interior to form the central nervous system. By marking and following the cells through these various movements, one can draw a fate map for the monolayer of cells on the surface of the blastoderm (Figure 21-28).

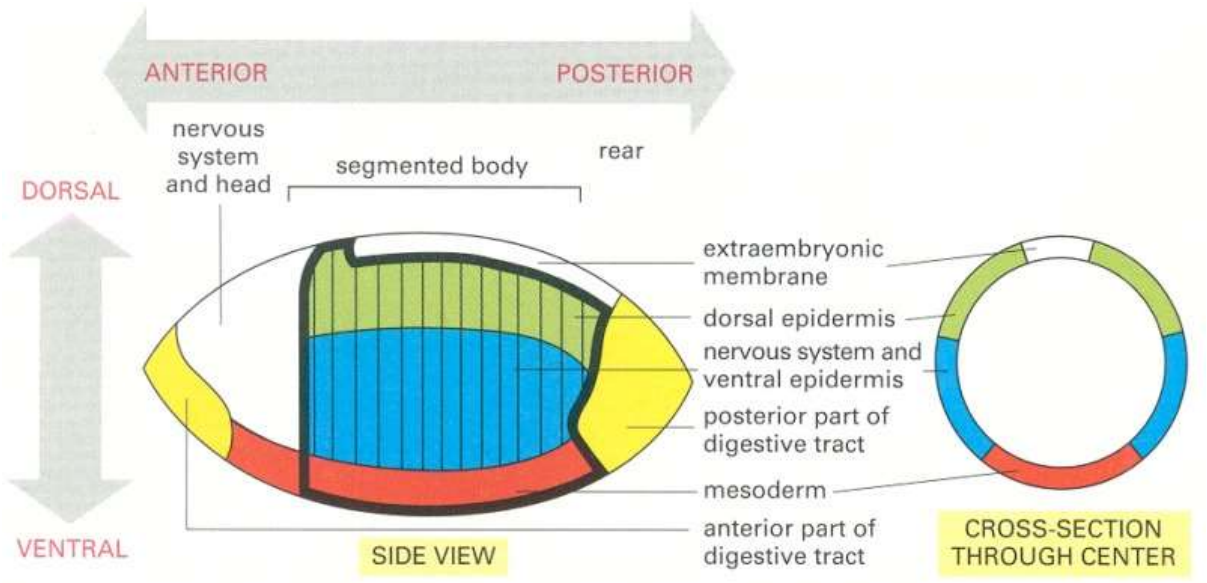


Figure 21-28 Fate map of a *Drosophila* embryo at the cellular blastoderm stage.The embryo is shown in side view and in cross section, displaying the relationship between the dorsoventral subdivision into future major tissue types and the anteroposterior pattern of future segments. A heavy line encloses the region that will form segmental structures. During gastrulation the cells along the ventral midline invaginate to form mesoderm, while the cells fated to form the gut invaginate near each end of the embryo.

As gastrulation nears completion, a series of indentations and bulges appear in the surface of the embryo, marking the subdivision of the body into segments along its anteroposterior axis (see Figure 21-25). Soon a fully segmented larva emerges, ready to start eating and growing. Within the body of the larva, small groups of cells remain apparently undifferentiated, forming structures called *imaginal discs*. These will grow as the larva grows, and eventually they will give rise to most of the structures of the adult body, as we shall see later.

A head end and a tail end, a ventral (belly) side and a dorsal (back) side, a gut, a nervous system, a series of body segments—these are all features of the basic body plan that *Drosophila* shares with many other animals, including ourselves. We begin our account of the mechanisms of *Drosophila* development by considering how this body plan is set up.

Genetic Screens Define Groups of Genes Required for Specific Aspects of Early Patterning

By carrying out a series of genetic screens based on saturation mutagenesis (see Chapter 8), it has been possible to amass a collection of *Drosophila* mutants that appears to include changes in a large proportion of the genes affecting development. Independent mutations in the same gene can be distinguished from mutations in separate genes by a complementation test, leading to a catalog of genes classified according to their mutant phenotypes. In such a catalog,

a group of genes with very similar mutant phenotypes will often code for a set of proteins that work together to perform a particular function.

Sometimes the developmental functions revealed by mutant phenotypes are those that one would expect; sometimes they are a surprise. A large-scale genetic screen focusing on early *Drosophila* development revealed that the key genes fall into a relatively small set of functional classes defined by their mutant phenotypes. Some—*the egg-polarity genes* (Figure 21-29)—are required to define the anteroposterior and dorsoventral axes of the embryo and mark out its two ends for special fates, by mechanisms involving interactions between the oocyte and surrounding cells in the ovary. Others, the *gap genes*, are required in specific broad regions along the anteroposterior axis of the early embryo to allow their proper development. A third category, the *pair-rule genes*, are required, more surprisingly, for development of alternate body segments. A fourth category, the *segment polarity genes*, are responsible for organizing the anteroposterior pattern of each individual segment.

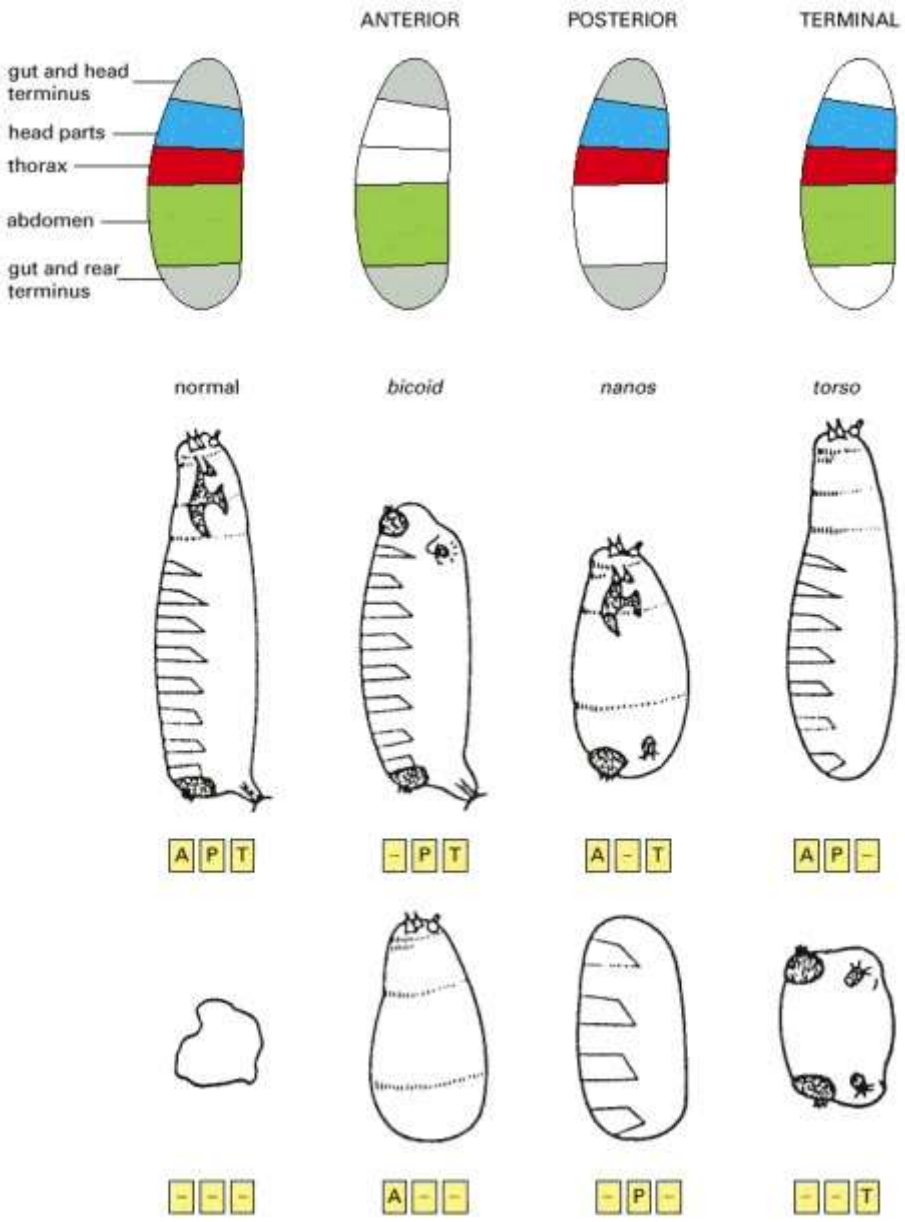


Figure 21-29 The domains of the anterior, posterior, and terminal systems of egg-polarity genes.The *upper* diagrams show the fates of the different regions of the egg/early embryo and indicate (in *white)* the parts that fail to develop if the anterior, posterior, or terminal system is defective. The *middle* row shows schematically the appearance of a normal larva and of mutant larvae that are defective in a gene of the anterior system (for example, *bicoid),* of the posterior system (for example, *nanos),* or of the terminal system (for example, *torso).* The *bottom* row of drawings shows the appearances of larvae in which none or only one of the three gene systems is functional. The lettering beneath each larva specifies which systems are intact (A P T for a normal larva, -P T for a larva where the anterior system is defective but the posterior and terminal systems are intact, and so on). Inactivation of a particular gene system causes loss of the corresponding set of body structures; the body parts that form correspond to the gene systems that remain functional. Note that larvae with a defect in the anterior system can still form terminal structures at their anterior end, but these are of the type normally found at the rear end of the body rather than the front of the head.

The discovery of these four systems of genes and the subsequent analysis of their functions (an enterprise that still continues) was a famous tour-de-force of developmental genetics. It has had a revolutionary impact on all of developmental biology by showing the way toward a systematic, comprehensive account of the genetic control of embryonic development. In this section, we shall summarize only briefly the conclusions relating to the earliest phases of *Drosophila* development, because these are insect-specific; we dwell at greater length on the parts of the process that illustrate more general principles.

Interactions of the Oocyte With Its Surroundings Define the Axes of the Embryo: the Role of the Egg-Polarity Genes

Surprisingly, the earliest steps of animal development are among the most variable, even within a phylum. A frog, a chicken, and a mammal, for example, even though they develop in similar ways later, make eggs that differ radically in size and structure, and they begin their development with different sequences of cell divisions and cell specialization events.

The style of early development that we have described for *C. elegans* is typical of many classes of animals. In contrast, the early development of *Drosophila* represents a rather extreme variant. The main axes of the future insect body are defined before fertilization by a complex exchange of signals between the unfertilized egg, or oocyte, and the follicle cells that surround it in the ovary (Figure 21-30). Then, in the syncytial phase following fertilization, an exceptional amount of patterning occurs in the array of rapidly dividing nuclei, before the first partitioning of the egg into separate cells. Here, there is no need for the usual forms of cell-cell communication involving transmembrane signaling; neighboring regions of the early *Drosophila* embryo can communicate by means of gene regulatory proteins and mRNA molecules that diffuse or are actively transported through the cytoplasm of the giant multinuclear cell.

n the stages before fertilization, the anteroposterior axis of the future embryo becomes defined by three systems of molecules that create landmarks in the oocyte (Figure 21-31). Following fertilization, each landmark serves as a beacon, providing a signal, in the form of a morphogen gradient, that organizes the developmental process in its neighbourhood. Two of these signals are generated from localized deposits of specific mRNA molecules. The future anterior end of the embryo contains a high concentration of mRNA for a gene regulatory protein called Bicoid; this mRNA is translated to produce Bicoid protein, which diffuses away from its source to form a concentration gradient with its maximum at the anterior end of the egg. The future posterior end of the embryo contains a high concentration of mRNA for a regulator of translation called Nanos, which sets up a posterior gradient in the same way. The third signal is generated symmetrically at both ends of the egg, by local activation of a transmembrane tyrosine kinase receptor called Torso. The activated receptor exerts its effects over a shorter range, marking the sites of specialized terminal structures that will form at the head and tail ends of the future larva and also defining the rudiments of the future gut. The three sets of genes responsible for these localized determinants are referred to as the anterior, posterior, and **terminal** sets of **egg-polarity** genes.

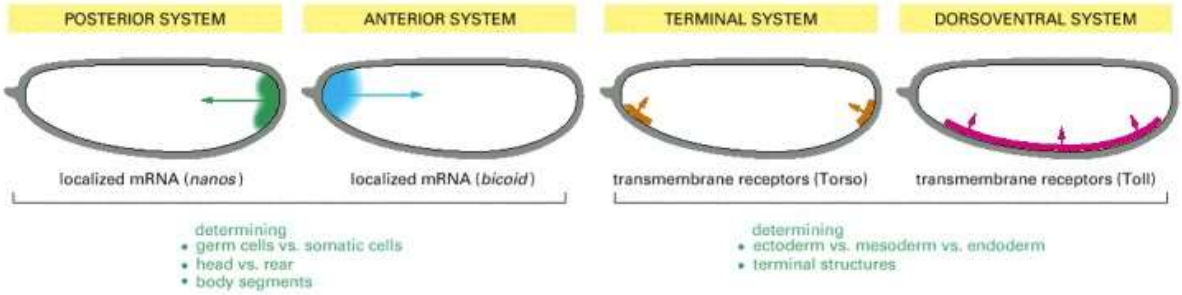


Figure 21-31 The organization of the four egg-polarity gradient systems.The receptors Toll and Torso are distributed all over the membrane; the coloring in the diagrams on the right indicates where they become activated by extracellular ligands.

A fourth landmark defines the dorsoventral axis (see Figure 21-31): a protein that is produced by follicle cells underneath the future ventral region of the embryo leads to localized activation of another transmembrane receptor, called Toll, in the oocyte membrane. The genes required for this function are called dorsoventral egg-polarity genes.

All the egg-polarity genes in these four classes are maternal-effect genes: it is the mother's genome, not the zygotic genome, that is critical. Thus, a fly whose chromosomes are mutant in both copies of the *bicoid* gene but who is born from a mother carrying one normal copy of *bicoid* develops perfectly normally, without any defects in the head pattern. However, if that daughter fly is a female no functional *bicoid* mRNA can be deposited into the anterior part of her own eggs, and all of these will develop into headless embryos regardless of the father's genotype.

Each of the four egg-polarity signals—provided by Bicoid, Nanos, Torso, and Toll—exerts its effect by regulating (directly or indirectly) the expression of genes in the nuclei of the blastoderm. The use of these particular molecules to organize the egg is not a general feature

of early animal development—indeed, only *Drosophila* and closely related insects possess a *bicoid* gene. And Toll has been coopted here for dorsoventral patterning; its more ancient and universal function is in the innate immune response.

Nevertheless, the egg-polarity system shows some highly conserved features. For example, the localization of *nanos* mRNA at one end of the egg is linked to, and dependent on, the localization of germ-cell determinants at that site, just as it is in *C. elegans*. Later in development, as the zygotic genome comes into play under the influence of the egg-polarity system, more similarities with other animal species become apparent. We shall use the dorsoventral system to illustrate this point.

The Dorsoventral Signalling Genes Create a Gradient of a Nuclear Gene Regulatory Protein

Localized activation of the Toll receptor on the ventral side of the egg controls the distribution of Dorsal, a gene regulatory protein inside the egg. The Dorsal protein belongs to the same family as the NF-κB gene regulatory protein of vertebrates (discussed in Chapter 15). Its Toll-regulated activity, like that of NF-κB, depends on its translocation from the cytoplasm, where it is held in an inactive form, to the nucleus, where it regulates gene expression. In the newly laid egg, both the *dorsal* mRNA (detected by *in situ* hybridization) and the protein it encodes (detected with antibodies) are distributed uniformly in the cytoplasm. After the nuclei have migrated to the surface of the embryo to form the blastoderm, however, a remarkable redistribution of the Dorsal protein occurs: dorsally the protein remains in the cytoplasm, but ventrally it is concentrated in the nuclei, with a smooth gradient of nuclear localization between these two extremes (Figure 21-32). The signal transmitted by the Toll protein controls this redistribution of Dorsal through a signalling pathway that is essentially the same as the Toll-dependent pathway involved in innate immunity.

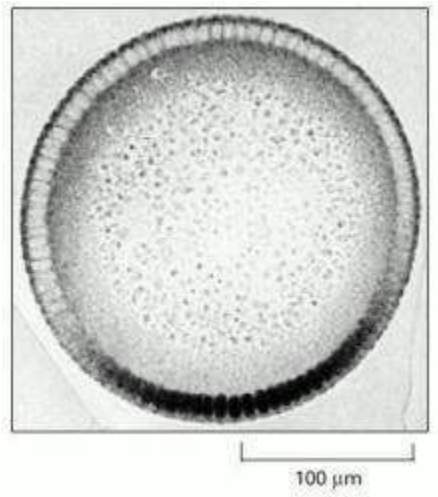


Figure 21-32 The concentration gradient of Dorsal protein in the nuclei of the blastoderm, as revealed by an antibody. Dorsally, the protein is present in the cytoplasm and absent from the nuclei; ventrally, it is depleted in the cytoplasm and concentrated in the nuclei.

Once inside the nucleus, the Dorsal protein turns on or off the expression of different sets of genes depending on its concentration. The expression of each responding gene depends on its regulatory DNA—specifically, on the number and affinity of the binding sites that this DNA contains for Dorsal and other regulatory proteins. In this way, the regulatory DNA can be said to *interpret* the positional signal provided by the Dorsal protein gradient, so as to define a dorsoventral series of territories—distinctive bands of cells that run the length of the embryo (Figure 21-33A). Most ventrally—where the concentration of Dorsal protein is highest—it switches on, for example, the expression of a gene called *twist* that is specific for mesoderm (Figure 21-34). Most dorsally, where the concentration of Dorsal protein is lowest, the cells switch on *decapentaplegic (dpp).* And in an intermediate region, where the concentration of Dorsal protein is high enough to repress *dpp* but too low to activate *twist,* the cells switch on another set of genes, including one called *short gastrulation (sog).*

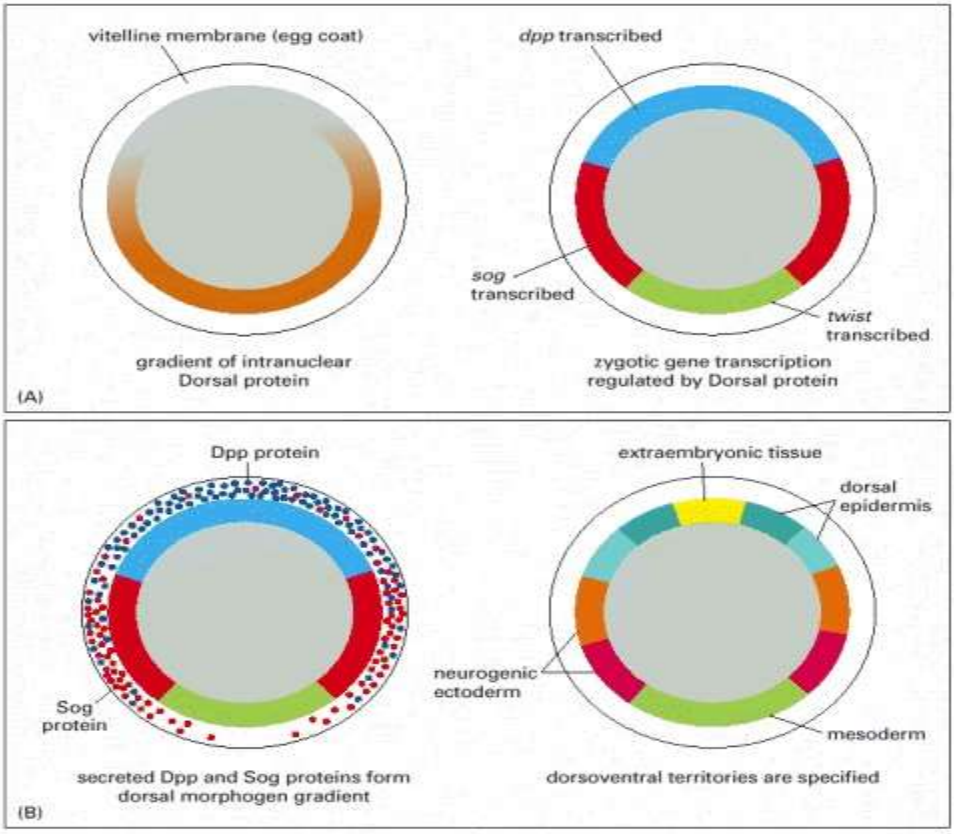


Figure 21-33 Morphogen gradients patterning the dorsoventral axis of the embryo.(A) The gradient of Dorsal protein defines three broad territories of gene expression, marked here by the expression of three representative genes—*dpp*, *sog*, and *twist*. (B) Slightly later, the cells expressing *dpp* and *sog* secrete, respectively, the signal proteins Dpp (a TGFβ family member) and Sog (an antagonist of Dpp). These two proteins diffuse and interact with one another (and with certain other factors) to set up a gradient of Dpp activity that guides a more detailed patterning process.

Dpp and Sog Set Up a Secondary Morphogen Gradient to Refine the Pattern of the Dorsal Part of the Embryo

Products of the genes directly regulated by the Dorsal protein generate in turn more local signals that define finer subdivisions of the dorsoventral axis. These signals act after cellularization, and take the form of conventional extracellular signalling molecules. In particular, *dpp* codes for the secreted Dpp protein, which forms a local morphogen gradient in the dorsal part of the embryo. The gene *sog*, meanwhile, codes for another secreted protein that is produced in the neurogenic ectoderm and acts as an antagonist of Dpp. The opposing diffusion gradients of these two proteins create a steep gradient of Dpp activity. The highest Dpp activity levels, in combination with certain other factors, cause development of the most dorsal tissue of all—extraembryonic membrane; intermediate levels cause development of dorsal ectoderm; and very low levels allow development of neurogenic ectoderm (Figure 21-33B).

Three Classes of Segmentation Genes Refine the Anterior- Posterior Maternal Pattern and Subdivide the Embryo

After the initial gradients of Bicoid and Nanos are created to define the anteroposterior axis, the **segmentation genes** refine the pattern. Mutations in any one of the segmentation genes alter the number of segments or their basic internal organization without affecting the global polarity of the embryo. Segmentation genes are expressed by subsets of cells in the embryo, so their products are the first components that the embryo's own genome, rather than the maternal genome, contributes to embryonic development. They are therefore called *zygotic-effect genes* to distinguish them from the earlier maternal-effect genes.

The segmentation genes fall into three groups according to their mutant phenotypes and the stages at which they act (Figure 21-36). First come a set of at least six **gap genes**, whose products mark out coarse subdivisions of the embryo. Mutations in a gap gene eliminate one or more groups of adjacent segments, and mutations in different gap genes cause different but partially overlapping defects. In the mutant *Krüppel,* for example, the larva lacks eight segments, from T1 to A5 inclusive.

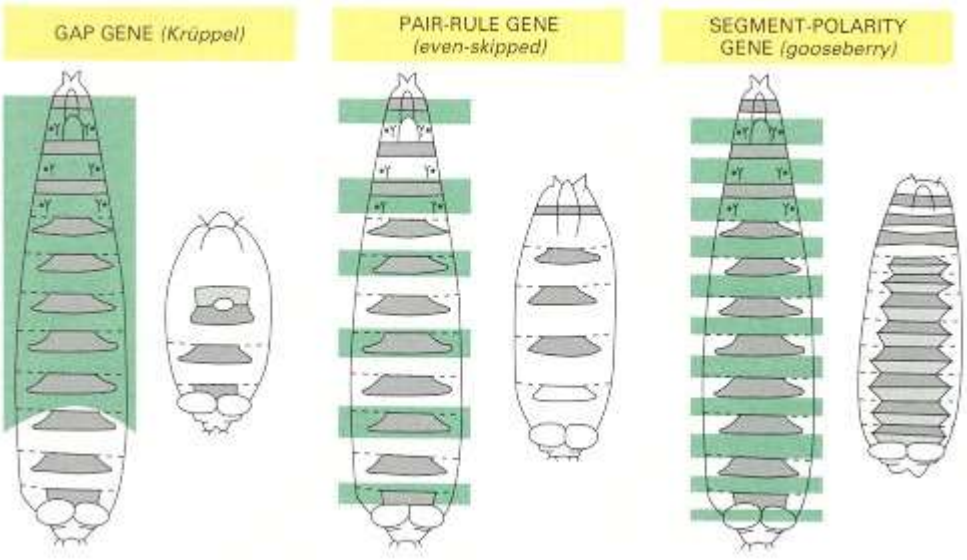


Figure 21-36 Examples of the phenotypes of mutations affecting the three types of segmentation genes. In each case the areas shaded in *green* on the normal larva *(left)* are deleted in the mutant or are replaced by mirror-image duplicates of the unaffected regions. By convention, dominant mutations are written with an initial capital letter and recessive mutations are written with a lower-case letter. Several of the patterning mutations of *Drosophila* are classed as dominant because they have a perceptible effect on the phenotype of the heterozygote, even though the characteristic major, lethal effects are recessive—that is, visible only in the homozygote.

The next segmentation genes to act are a set of eight **pair-rule genes**. Mutations in these cause a series of deletions affecting alternate segments, leaving the embryo with only half as many segments as usual. While all the pair-rule mutants display this two-segment periodicity, they differ in the precise positioning of the deletions relative to the segmental or parasegmental borders. The pair-rule mutant *even-skipped (eve)*, for example, which is discussed in Chapter 9, lacks the whole of each odd-numbered parasegment, while the pair-rule mutant *fushi tarazu (ftz)* lacks the whole of each even-numbered parasegment, and the pair-rule mutant *hairy* lacks a series of regions that are of similar width but out of register with the parasegmental units.

Finally, there are at least 10 **segment-polarity genes**. Mutations in these genes produce larvae with a normal number of segments but with a part of each segment deleted and replaced by a mirror-image duplicate of all or part of the rest of the segment. In *gooseberry* mutants, for example, the posterior half of each segment (that is, the anterior half of each parasegment) is replaced by an approximate mirror image of the adjacent anterior half-segment (see Figure 21-36).

We see later that, in parallel with the segmentation process, a further set of genes, the *homeotic selector genes,* serve to define and preserve the differences between one segment and the next. The phenotypes of the various segmentation mutants suggest that the segmentation genes form a coordinated system that subdivides the embryo progressively into smaller and smaller domains along the anteroposterior axis, distinguished by different patterns of gene expression. Molecular genetics has helped to reveal how this system works.

The Localized Expression of Segmentation Genes Is Regulated by a Hierarchy of Positional Signals

About three-quarters of the segmentation genes, including all of the gap genes and pair-rule genes, code for gene regulatory proteins. Their actions on one another and on other genes can therefore be observed by comparing gene expression in normal and mutant embryos. By using appropriate probes to detect the gene transcripts or their protein products, one can, in effect, take snapshots as genes switch on and off in changing patterns. Repeating the process with mutants that lack a particular segmentation gene, one can begin to dissect the logic of the entire gene control system.

The products of the egg-polarity genes provide the global positional signals in the early embryo. These cause particular gap genes to be expressed in particular regions. The products of the gap genes then provide a second tier of positional signals that act more locally to regulate finer details of patterning through the expression of yet other genes, including the pair-rule genes (Figure 21-37). The pair-rule genes in turn collaborate with one another and with the gap genes to set up a regular periodic pattern of expression of segment-polarity genes, and the segment-polarity genes collaborate with one another to define the internal pattern of each individual segment. The strategy, therefore, is one of sequential induction (see Figure 21-15). By the end of the process, the global gradients produced by the egg-polarity genes have triggered the creation of a fine-grained pattern through a hierarchy of sequential, progressively more local, positional controls. Because the global positional signals that start the process do not have to directly specify fine details, the individual cell nuclei do not have to be governed with extreme precision by small differences in the concentration of these signals. Instead, at each step in the sequence, new signals come into play, providing substantial localized differences of concentration to define new details. Sequential induction is thus a robust strategy. It works reliably to produce fly embryos that all have the same pattern, despite the essential imprecision of biological control systems, and despite variations in conditions such as the temperature at which the fly develops.

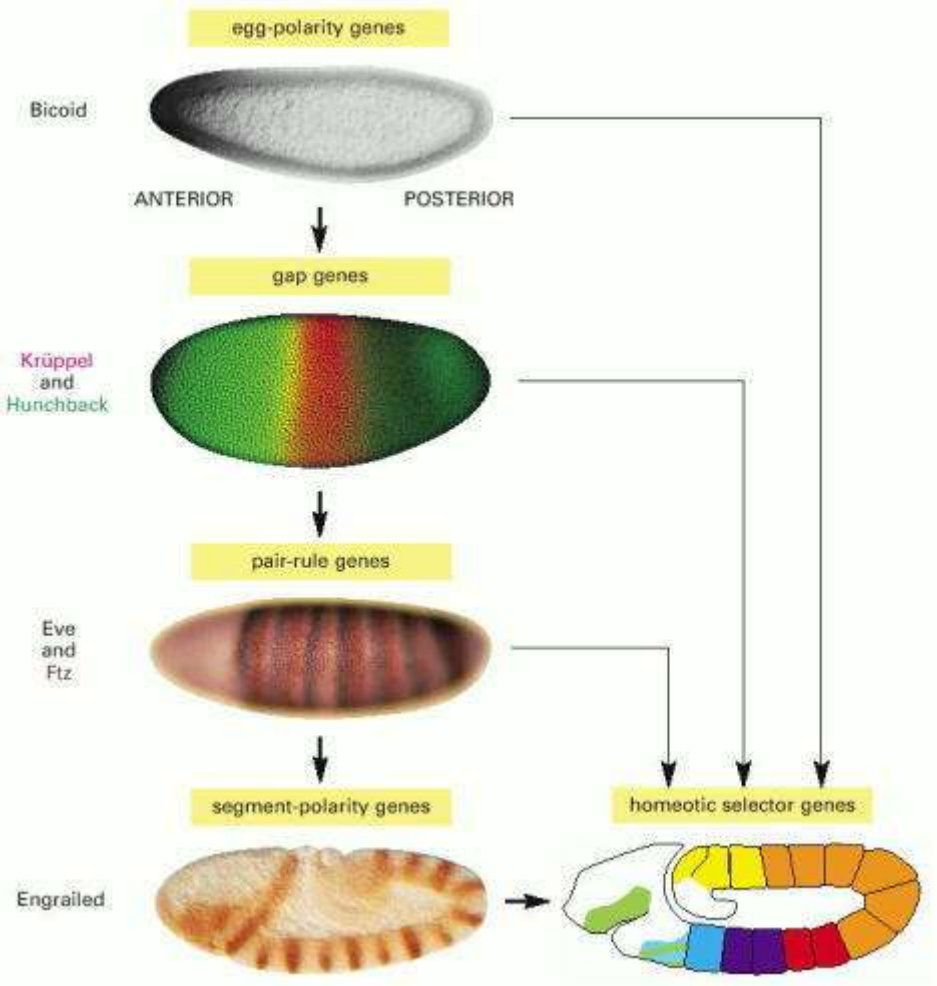


Figure 21-37 The regulatory hierarchy of egg-polarity, gap, segmentation, and homeotic selector genes. The photographs show expression patterns of representative examples of genes in each category, revealed by staining with antibodies against the protein products. The homeotic selector genes, discussed below, define the lasting differences between one segment and the next.

Egg-Polarity, Gap, and Pair-Rule Genes Create a Transient Pattern That Is Remembered by Other Genes

Within the first few hours after fertilization, the gap genes and the pair-rule genes are activated one after another. Their mRNA products appear first in patterns that only approximate the final picture; then, within a short time—through a series of interactive adjustments—the fuzzy initial distribution of gene products resolves itself into a regular, crisply defined system of stripes (Figure 21-39). But this system itself is unstable and transient. As the embryo proceeds through gastrulation and beyond, the regular segmental pattern of gap and pair-rule gene products disintegrates. Their actions, however, have stamped a permanent set of labels—positional values—on the cells of the blastoderm. These positional labels are recorded in the persistent activation of certain of the segment-polarity genes and of the homeotic selector

genes, which serve to maintain the segmental organization of the larva and adult. The segment-polarity gene *engrailed* provides a good example. Its RNA transcripts are seen in the cellular blastoderm in a series of 14 bands, each approximately one cell wide, corresponding to the anteriormost portions of the future parasegments (Figure 21-40).

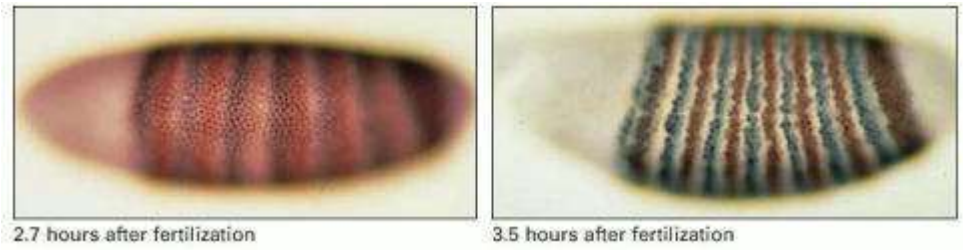


Figure 21-39 The formation of *ftz* and *eve* stripes in the *Drosophila* blastoderm *ftz* and *eve* are both pair-rule genes. Their expression patterns (shown in *brown* for *ftz* and in *gray* for *eve*) are at first blurred but rapidly resolve into sharply defined stripes.

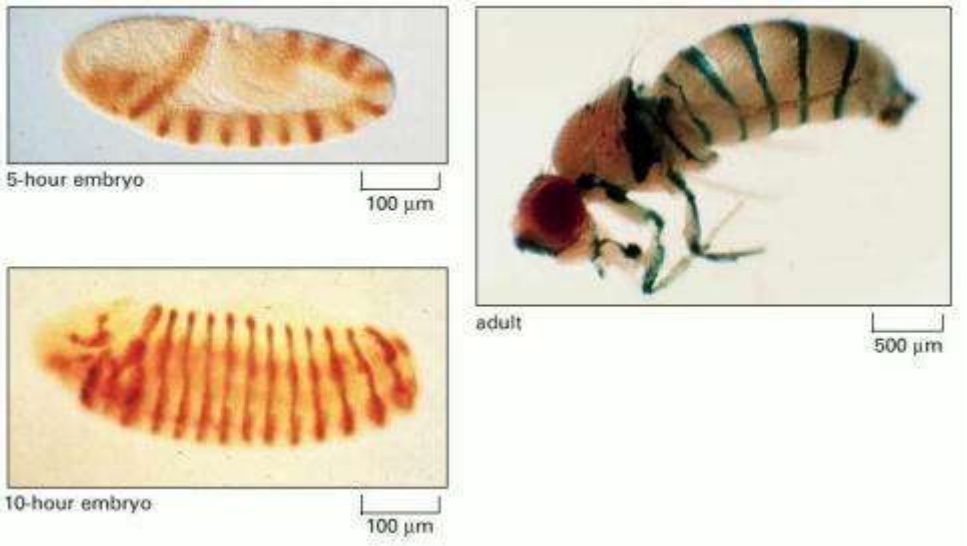


Figure 21-40 The pattern of expression of *engrailed*, a segment-polarity gene. The *engrailed* pattern is shown in a 5-hour embryo (at the extended germ-band stage), a 10-hour embryo, and an adult (whose wings have been removed in this preparation). The pattern is revealed by an antibody *(brown)* against the Engrailed protein (for the 5- and 10-hour embryos) or (for the adult) by constructing a strain of *Drosophila* containing the control sequences of the *engrailed* gene coupled to the coding sequence of the reporter *LacZ*, whose product is detected histochemically through the *blue* product of a reaction that it catalyses. Note that the *engrailed* pattern, once established, is preserved throughout the animal's life.

The segment-polarity genes are expressed in patterns that repeat from one parasegment to the next, and their bands of expression appear in a fixed relationship to the bands of expression of the pair-rule genes that help to induce them. However, the production of this pattern within each parasegment depends on interactions among the segment-polarity genes themselves. These interactions occur at stages when the blastoderm has already become fully partitioned into separate cells, so that cell-cell signalling of the usual sort has to come into play. A large subset of the segment-polarity genes code for components of two signal transduction pathways, the Wnt pathway and the Hedgehog pathway, including the secreted signal proteins Wingless (a Wnt family member) and Hedgehog. These are expressed in different bands of cells that serve as signalling centers within each parasegment, and they act to maintain and refine the expression of other segment-polarity genes. Moreover, although their initial expression is determined by the pair-rule genes, the two signalling proteins regulate one another's expression in a mutually supportive way, and they proceed to help trigger expression of genes such as *engrailed* in precisely the correct sites.

The *engrailed* expression pattern will persist throughout life, long after the signals that organized its production have disappeared (see Figure 21-40). This example illustrates not only the progressive subdivision of the embryo by means of more and more narrowly localized signals, but also the transition between the transient signalling events of early development and the later stable maintenance of developmental information.

Besides regulating the segment-polarity genes, the products of pair-rule genes collaborate with the products of gap genes to cause the precisely localized activation of a further set of spatial labels—the homeotic selector genes. It is the homeotic selector genes that permanently distinguish one parasegment from another. In the next section we examine these selector genes in detail and consider their role in cell memory.

Evolution of Homeotic genes; Homeo domains; Hox genes & HOM-c genes:

In 1894, William Bateson coined the word **homeosis** to describe the situation in which "something has been changed into the likeness of something else" (Lewis 1994). Bateson was attempting to provide evidence in support of Darwin's theory of evolution and homeotic variations seemed to Bateson to be the kind of dramatic changes that could explain how evolution occurred. E. B. Lewis (1994) concluded that homeosis provided a rich legacy: "Besides giving us the homeobox, it has opened up a completely new approach to the study of development. And over the past 15 years, it as led to the realization that the body plan of most animals, and presumably of plants as well, is controlled by a set of master regulatory genes, first identified by their homeotic mutations."

The periodic pattern of body segments generated by segmentation genes (gap genes, pair-rule genes, and segment-polarity genes) has to be converted into segments with wings, legs, and antennae (Figure 4.7). Thus, in insects, thoracic segment 2 is different from thoracic segment 3 and abdominal segment 2 will be different from the terminal abdominal segments, which typically have genital structures. This fine-tuning is determined by **homeotic** or **Hox genes**.

The homeobox consists of ≈180 bp that is translated into a 60-amino-acid domain. The sequences of the different homeoboxes are nearly identical and they mediate the binding of homeotic proteins to specific DNA sequences and thus regulate the expression of many

downstream genes. It has been proposed that just two homeotic genes, *even-skipped*$^{+}$ and *Fushi tarazu*$^{+}$, directly control the expression of the majority of genes in the *Drosophila* genome (Mannervik 1999). Homeodomain proteins occur in all eukaryotes, where they perform important functions during development.

Since the first homeobox sequence was isolated from the *Antennapedia*$^{+}$ gene in late 1983, it has been used as a probe to identify and isolate previously unknown homeotic genes from *Drosophila*. Furthermore, because the homeobox is evolutionarily conserved, this *Drosophila* sequence was used as a probe to identify homeotic genes from other species, including humans (Gehring 1985).The products of Hox genes are Hox proteins. Hox proteins are a subset of the homeodomain-containing transcription factors, which are proteins that are capable of binding to specific nucleotide sequences on the DNA called enhancers where they either activate or repress genes. The same Hox protein can act as a repressor at one gene and an activator at another. The ability of Hox proteins to bind DNA is conferred by a part of the protein referred to as the homeodomain. The homeodomain is a 60-amino-acid-long DNA-binding domain (encoded by its corresponding 180-base-pair DNA sequence, the homeobox). This amino acid sequence folds into a "helix-turn-helix" (i.e. homeodomain fold) motif that is stabilized by a third helix. The consensus polypeptide chain is:

```
         Helix 1         Helix 2         Helix 3/4
      _______________   ___________   ___________________
RRRKRTAYTRYQLLELEKEFLFNRYLTRRRRIELAHSLNLTERHIKIWFQNRRMKWK
KEN
....|....|....|....|....|....|....|....|....|....|....|
        10        20        30        40        50        60
```

In Drosophila, like all insects, has eight Hox genes. These are clustered into two complexes, both of which are located on chromosome 3. The Antennapedia complex (not to be confused with the *Antp* gene) consists of five genes: labial (*lab*), proboscipedia (*pb*), deformed (*Dfd*), sex combs reduced (*Scr*), and Antennapedia (*Antp*). The Bithorax complex, named after the Ultrabithorax gene, consists of the remaining three genes: Ultrabithorax (*Ubx*), abdominal-A (*abd-A*) and abdominal-B (*abd-B*).

a. Labial

The *lab* gene is the most anteriorly expressed gene. It is expressed in the head, primarily in the intercalary segment (an appendageless segment between the antenna and mandible), and also in the midgut. Loss of function of *lab* results in the failure of the *Drosophila* embryo to internalize the mouth and head structures that initially develop on the outside of its body (a process called head involution). Failure of head involution disrupts or deletes the salivary glands and pharynx. The *lab* gene was initially so named because it disrupted the labial appendage; however, the lab gene is not expressed in the labial segment, and the labial appendage phenotype is likely a result of the broad disorganization resulting from the failure of head involution

b. Proboscipedia

The *pb* gene is responsible for the formation of the labial and maxillary palps. Some evidence shows *pb*interacts with *Scr*.

c. Deformed

The *Dfd* gene is responsible for the formation of the maxillary and mandibular segments in the larval head.The mutant phenotypes of *Dfd* are similar to those of labial. Loss of function of *Dfd* in the embryo results in a failure of head involution (see labial gene), with a loss of larval head structures. Mutations in the adult have either deletions of parts of the head or transformations of head to thoracic identity.

d. Sex combs reduced

The *Scr* gene is responsible for cephalic and thoracic development in *Drosophila* embryo and adult.

e. Antennapedia

The second thoracic segment, or T2, develops a pair of legs and a pair of wings. The *Antp* gene specifies this identity by promoting leg formation and allowing (but not directly activating) wing formation. A dominant *Antp* mutation, caused by a chromosomal inversion, causes *Antp* to be expressed in the antennal imaginal disc, so that, instead of forming an antenna, the disc makes a leg, resulting in a leg coming out of the fly's head.

Figure: Wild type (left), Antennapedia mutant (right)

Ultrabithorax

The third thoracic segment, or T3, bears a pair of legs and a pair of halteres (highly reduced wings that function in balancing during flight). *Ubx*patterns T3 largely by repressing genes involved in wing formation. The wing blade is composed of two layers of cells that adhere tightly to one another, and are supplied with nutrient by several wing veins. One of the many genes that *Ubx* represses is blistered, which activates proteins involved in cell-cell adhesion, and spalt, which patterns the placement of wing veins. In *Ubx* loss-of-function mutants, *Ubx* no longer represses wing genes, and the halteres develop as a second pair of wings, resulting in the famous four-winged flies. When *Ubx* is misexpressed in the second thoracic segment, such as occurs in flies with the "Cbx" enhancer mutation, it represses wing genes, and the wings develop as halteres, resulting in a four-haltered fly.

Abdominal-A

In *Drosophila*, *abd-A* is expressed along most of the abdomen, from abdominal segments 1 (A1) to A8. Expression of *abd-A* is necessary to specify the identity of most of the abdominal segments. A major function of *abd-A* in insects is to repress limb formation. In *abd-A* loss-of-function mutants, abdominal segments A2 through A8 are transformed into an identity more like A1. When *abd-A* is ectopically expressed throughout the embryo, all segments anterior of A4 are transformed to an A4-like abdominal identity.[7] The *abd-A gene* also affects the pattern of cuticle generation in the ectoderm, and pattern of muscle generation in the mesoderm.

Abdominal-B

Gene *abd-B* is transcribed in two different forms, a regulatory protein, and a morphogenic protein. Regulatory *abd-B* suppress embryonic ventral epidermal structures in the eighth and ninth segments of the *Drosophila* abdomen. Both the regulatory protein and the morphogenic protein are involved in the development of the tail segment.

Classification of Hox proteins

Proteins with a high degree of sequence similarity are also generally assumed to exhibit a high degree of functional similarity, i.e. Hox proteins with identical homeodomains are assumed to have identical DNA-binding properties (unless additional sequences are known to influence DNA-binding). To identify the set of proteins between two different species that are most likely to be most similar in function, classification schemes are used. For Hox proteins, three different classification schemes exist: phylogenetic inference based, synteny-based, and sequence similarity-based. The three classification schemes provide conflicting information for Hox proteins expressed in the middle of the body axis (*Hox6-8*and *Antp*, *Ubx* and *abd-A*). A combined approach used phylogenetic inference-based information of the different species and plotted the protein sequence types onto the phylogenetic tree of the species. The approach identified the proteins that best represent ancestral forms (*Hox7* and *Antp*) and the proteins that represent new, derived versions (or were lost in an ancestor and are now missing in numerous species).

Genes regulated by Hox proteins

Hox genes act at many levels within developmental gene hierarchies: at the "executive" level they regulate genes that in turn regulate large networks of other genes (like the gene pathway that forms an appendage). They also directly regulate what are called realisator genes or effector genes that act at the bottom of such hierarchies to ultimately form the tissues, structures, and organs of each segment. Segmentation involves such processes as morphogenesis (differentiation of precursor cells into their terminal specialized cells), the tight association of groups of cells with similar fates, the sculpting of structures and segment boundaries via programmed cell death, and the movement of cells from where they are first born to where they will ultimately function, so it is not surprising that the target genes of Hox genes promote cell division, cell adhesion, apoptosis, and cell migration.

Examples of targets

Organism	Target gene	Normal function of target gene	Regulated by
Drosophila	distal-less	activates gene pathway for limb formation	ULTRABITHORAX[14] (represses distal-less)
	distal-less	activates gene pathway for limb formation	ABDOMINAL-A[14] (represses distal-less)
	decapentaplegic	triggers cell shape changes in the gut that are required for normal visceral morphology	ULTRABITHORAX[15] (activates decapentaplegic)
	reaper	Apoptosis: localized cell death creates the segmental boundary between the maxilla and mandible of the head	DEFORMED[16] (activates reaper)
	decapentaplegic	prevents the above cell changes in more posterior positions	ABDOMINAL-B[15] (represses decapentaplegic)

Enhancer sequences bound by homeodomains

The DNA sequence bound by the homeodomain protein contains the nucleotide sequence TAAT, with the 5' terminal T being the most important for binding. This sequence is conserved in nearly all sites recognized by homeodomains, and probably distinguishes such locations as DNA binding sites. The base pairs following this initial sequence are used to distinguish between homeodomain proteins, all of which have similar recognition sites. For instance, the nucleotide following the TAAT sequence is recognized by the amino acid at position 9 of the homeodomain protein. In the maternal protein Bicoid, this position is occupied by lysine, which recognizes and binds to the nucleotide guanine. In Antennapedia, this position is occupied by glutamine, which recognizes and binds to adenine. If the lysine in Bicoid is replaced by glutamine, the resulting protein will recognize Antennapedia-binding enhancer sites.

However, all homeodomain-containing transcription factors bind essentially the same DNA sequence. The sequence bound by the homeodomain of a Hox protein is only six nucleotides long, and such a short sequence would be found at random many times throughout the genome, far more than the number of actual functional sites. Especially for Hox proteins, which produce such dramatic changes in morphology when misexpressed, this raises the question of how each transcription factor can produce such specific and different outcomes if they all bind the same sequence. One mechanism that introduces greater DNA sequence specificity to Hox proteins

is to bind protein cofactors. Two such Hox cofactors are Extradenticle (Exd) and Homothorax (Hth). Exd and Hth bind to Hox proteins and appear to induce conformational changes in the Hox protein that increase its specificity.

Regulation of Hox genes

Just as Hox genes regulate realisator genes, they are in turn regulated themselves by gap genes and pair-rule genes, which are in their turn regulated by maternally-supplied mRNA. This results in a transcription factor cascade: maternal factors activate gap or pair-rule genes; gap and pair-rule genes activate Hox genes; then, finally, Hox genes activate realisator genes that cause the segments in the developing embryo to differentiate. Regulation is achieved via protein concentration gradients, called morphogenic fields. For example, high concentrations of one maternal protein and low concentrations of others will turn on a specific set of gap or pair-rule genes. In flies, stripe 2 in the embryo is activated by the maternal proteins Bicoid and Hunchback, but repressed by the gap proteins Giant and Kruppel. Thus, stripe 2 will only form wherever there is Bicoid and Hunchback, but *not* where there is Giant and Kruppel.

MicroRNA strands located in Hox clusters have been shown to inhibit more anterior hox genes ("posterior prevalence phenomenon"), possibly to better fine tune its expression pattern. Non-coding RNA (ncRNA) has been shown to be abundant in Hox clusters. In humans, 231 ncRNA may be present. One of these, HOTAIR, silences in trans (it is transcribed from the HOXC cluster and inhibits late HOXD genes) by binding to Polycomb-group proteins (PRC2). The chromatin structure is essential for transcription but it also requires the cluster to loop out of the chromosome territory.

Homeotic mutants may have segments that are transformed dramatically. For example, antennal segments may be transformed into leg-like structures, and metathoracic segments with halteres may be transformed into mesothoracic segments with a set of wings. The four-winged *D. melanogaster* is the result of combining three separate mutated genes in one fly! Normally, of course, a pair of wings is found on the second thoracic segment and a pair of balancing organs, called halteres, is on the third. However, this fly has two essentially normal second thoraces (and no third thoracic segment) because the combined effect of the three mutations is to transform the third thoracic segment into the second without affecting any other parts of the fly.

Lewis, E., (1978) proposed a combinatorial model that assumes each insect segment is specified by a unique combination of homeotic genes that are expressed in that particular segment. Thus, the fewest number of homeotic genes would be required in thoracic segment 2, which would be the prototypical segment, and progressively more genes would be active in the more-posterior segments. Although this model has been modified, it provided a useful conceptual framework for investigating *Drosophila* development.

Homeotic genes have some unusual characteristics. First, several homeotic genes seem to be very large relative to most other genes in *Drosophila*. For example, the *Antennapedia*$^+$ primary gene transcript is ≈100 kb long and the *Ultrabithorax*$^+$ transcript is ≈75 kb. However, after the introns are spliced out, the remaining sequences are only a few kilobases. Many of the exons in homeotic genes seem to encode protein domains with distinct structural or enzymatic functions. As a result, alternative splicing patterns in large genes, such as the *Antennapedia*$^+$ and *bithorax*$^+$gene complexes, may allow organisms to adapt one basic

protein structure to different, but related, developmental uses. By adding or subtracting functional protein domains encoded by optional exons, the structural and enzymatic properties of the homeotic gene product can be modified and the ability of the protein to interact with other cellular components can be altered as development proceeds.

Homeotic Gene Cluster (HOM-c)

Drosophila has two homeotic gene clusters: the ANT-C (*Antennapedia* complex) is responsible for segmental identity in the head and anterior thorax and the BX-C (*Bithorax* complex) which is responsible for segmental identity in the posterior thorax and abdomen.

These two gene clusters found on chromosome 3 are found in one cluster in more primitive insects, called the HOM-C (homeotic gene complex). The general case is that there is only one homeotic gene cluster in insects and in the evolution of *Drosophila* it was separated into two clusters.

Summary

The fly *Drosophila* has been the foremost model organism for study of the genetics of animal development. Like other insects, it begins its development with a series of nuclear divisions generating a syncytium, and a large amount of early patterning occurs in this single giant multinucleate cell. The pattern originates with asymmetry in the egg, organized both by localized deposits of mRNA inside the egg and by signals from the follicle cells around it. Positional information in the multinucleate embryo is supplied by four intracellular gradients that are set up by the products of four groups of maternal-effect genes called egg-polarity genes. These control four distinctions fundamental to the body plan of animals: dorsal versus ventral, endoderm versus mesoderm and ectoderm, germ cells versus somatic cells, and head versus rear.

The egg-polarity genes operate by setting up graded distributions of gene regulatory proteins in the egg and early embryo. The gradients along the anteroposterior axis initiate the orderly expression of gap genes, pair-rule genes, segment-polarity genes, and homeotic selector genes. These, through a hierarchy of interactions, become expressed in some regions of the embryo and not others, progressively subdividing the blastoderm into a regular series of repeating modular units called segments. The complex patterns of gene expression reflect the modular organization of the regulatory *DNA*, with separate enhancers of an individual gene responsible for separate parts of its expression pattern.

The segment-polarity genes come into play toward the end of the segmentation process, soon after the syncytium has become partitioned into separate cells, and they control the internal patterning of each segment through cell-cell signalling via the Wnt (Wingless) and Hedgehog pathways. This leads to persistent localized activation of genes such as engrailed, giving cells a remembered record of their anteroposterior address within the segment. Meanwhile, a new cell-cell signalling gradient is also set up along the dorsoventral axis, with the TGFβ family member Decapentaplegic (Dpp) and its antagonist, Short gastrulation, acting as the

morphogens. This gradient helps to refine the assignment of different characters to cells at different dorsoventral levels. Homologous proteins are also known to control the patterning of the ventrodorsal axis in vertebrates.

Probable questions:

1. Describe different stages of life cycle of Drosophila.
2. How dorsoventral axis is determined in Drosophila ?
3. How anterior posterior side is determined in Drosophila?
4. State the function of dorsal protein.
5. What is the role of pair rule gene in Drosophila development?
6. What is the role of gap gene in Drosophila development?
7. What is the role of segment polarity gene in Drosophila development?
8. What is Hox gene? What is Homeo domain?
9. What is the role of Dpp and Sog protein in determination of pattern of the dorsal part of the Drosophila embryo ?
10. How Hox genes are regulated ?
11. What is Homeotic gene cluster?

Suggested Readings:

1. Developmental biology. Gilbert, S. F., & Barresi, M. J. F. (2016).

2. Molecular Biology of the Cell – by Bruce Alberts

3. Molecular Cell Biology by Lodish, Fourth Edition.

Unit-II

Structural genomics: High resolution chromosome map-RFLP RAPD, fluorescence in situ hybridization; radiation hybrid mapping ; physical mapping of genomes ; genome sequencing

Objective: In this unit we will discuss about different aspects of structural genomics. RFLP-RAPD mapping will be discussed . FISH technique and radiation hybrid mapping will also be discussed. Genome sequencing will also be discussed in detail.

A. Molecular Markers in Physical Mapping:

Different types of molecular markers are used to understand and ascertain relationship in different organisms/individuals as well as to detect or diagnose character. These markers are to locate certain characteristics on the gel (banding pattern) which can be used to detect a specific character/defect in the genome. Unlike genetic mapping, physical mapping is not to locate the genes/characters on a genome, but to create a unique pattern by processing the genomic DNA.

There are several molecular markers available which are used depending upon the objective of the work and facilities available at the centre. Use of these markers to create maps (e.g., electrophoretic patterns) of an organism is known as 'physical mapping'. Molecular markers used in physical mapping are described below. New technologies are also developed simultaneously to resolve biological problems and help legal proceedings.

Restriction Fragment Length Polymorphism (RFLP):

RFLP is a method used by molecular biologists to follow a particular sequence of DNA as it is passed on to other cells. RFLPs can be used in many different settings to accomplish different objectives. RFLPs can be used in paternity cases or criminal cases to determine the source of a DNA sample. RFLPs can be used to determine the disease status of an individual. RFLPs can be used to measure recombination rates which can lead to a genetic map with the distance between RFLP loci measured in centiMorgans.

RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. It is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. Most RFLP markers are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific.

An RFLP probe is a labelled DNA sequence that hybridizes with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Short, single- or low-copy genomic DNA or cDNA clones are typically used as RFLP probes. The RFLP probes are

frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, hereditary disease diagnostics, etc.) (Fig. 21.5).

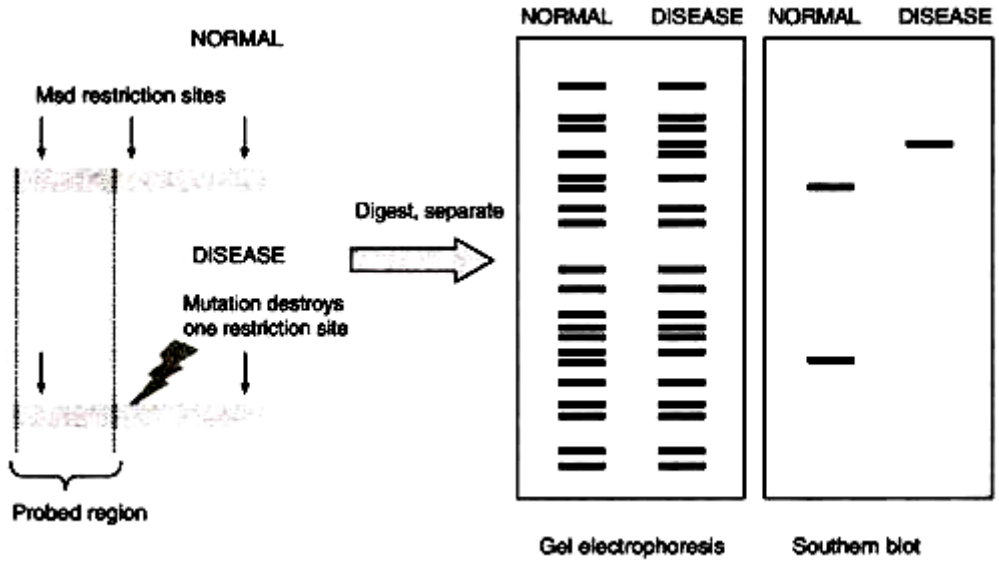


Fig. 21.5. Disease diagnosis by RFLP.

i. Procedure:

Usually, DNA from an individual specimen is first extracted and purified. Purified DNA may be amplified by polymerase chain reaction (PCR), The DNA is then cut into restriction fragments using suitable endonucleases, which only cut the DNA molecule where there are specific DNA sequences, termed recognition sequence or restriction sites that are recognized by the enzymes.

These sequences are specific to each enzyme, and may be either four, six, eight, ten or twelve base pairs in length. The more base pairs there are in the restriction site, the more specific it is and the lower the probability that it will find a place to be cut. The restriction fragments are then separated according to length by agarose gel electrophoresis. The resulting gel may be enhanced by Southern blotting. Alternatively, fragments may be visualized by pre-treatment or post-treatment of the agarose gel, using methods such as ethidium bromide staining or silver staining respectively.

RFLPs have provided valuable information in many areas of biology, including: screening human DNA for the presence of potentially deleterious genes (Fig. 21.6). Providing evidence to establish the innocence of or a probability of the guilt of, a crime suspect by DNA "fingerprinting". The distance between the locations cut by restriction enzymes (the restriction sites) varies between individuals, due to insertions, deletions or trans-versions.

This causes the length of the fragments to vary, and the position of certain amplicons differs between individuals (thus polymorphism). This can be used to genetically tell individuals apart. It can also show the genetic relationship between individuals, because children inherit genetic

elements from their parents. Mitochondrial DNA RFLP analyses can lead to the determination of maternal relationships.

Fragments may also be used to determine relationships among and between species by comparison of the resulting haplotypes (abridged for 'haploid genotype'). RFLP is a technique used in marker assisted selection. Terminal Restriction Fragment Length Polymorphism (TRFLP or sometimes T-RFLP) is a molecular biology technique initially developed for characterizing bacterial communities in mixed-species samples. The technique has also been applied to other groups including soil fungi.

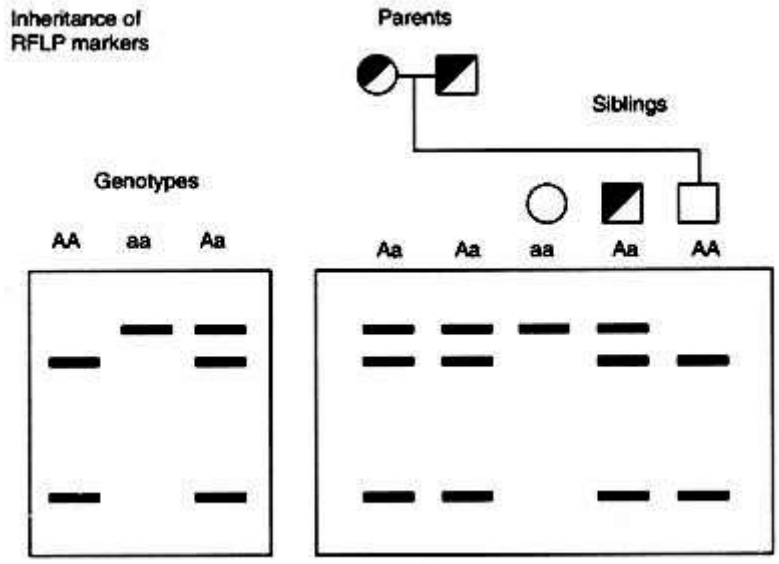


Fig. 21.6. Inheritance of RFLP markers.

The technique works by PCR amplification of DNA using primer pairs that have been labelled with fluorescent tags. The PCR products are then digested using RFLP enzymes and the resulting patterns visualized using a DNA sequencer. The results are analyzed either by simply counting and comparing bands or peaks in the TRFLP profile, or by matching bands from one or more TRFLP runs to a database of known species.

ii. Measurement of distance between two RFLP loci:

To calculate the genetic distance between two loci, you need to be able to observe recombination. Traditionally, this was performed by observing phenotypes but with RFLP analysis, it is possible to measure the genetic distance between two RFLP loci whether they are a part of genes or not. Let's look at a simple example in fruit flies. Two RFLP loci with two RFLP bands possible at each locus (Fig. 21.7).

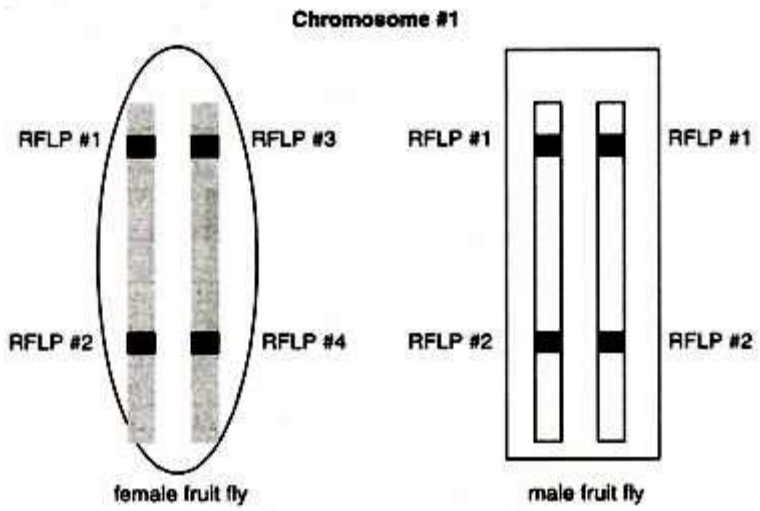


Fig. 21.7. RFLP loci on male and female chromosome.

These loci are located on the same chromosome for the female (left) and the male (right). The upper locus can produce two different bands called 1 and 3. The lower locus can produce bands called 2 or 4. The male is homozygous for band 1 at the upper locus and 2 for the lower locus. The female is heterozygous at both loci. Their RFLP banding patterns can be seen on the Southern blot below (Fig. 21.8).

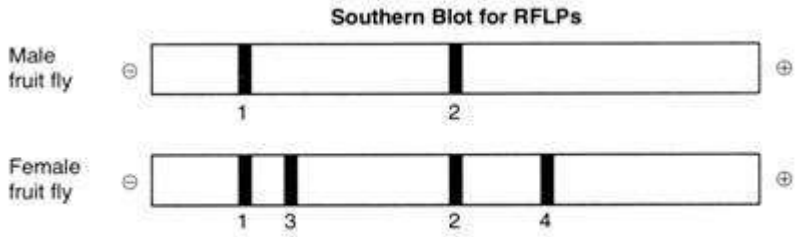


Fig. 21.8. RFLP loci on gel of male and female chromosome.

The male can only produce one type of gamete (1 and 2) but the female can produce four different gametes. Two of the possible four are called parental because they carry both RFLP bands from the same chromosome; 1 and 2 from the left chromosome or 3 and 4 from the right chromosome. The other two chromosomes are recombinant because recombination has occurred between the two loci and thus the RFLP bands are mixed so that 1 is now linked to 4 and 3 is linked to 2.

Type of chromatid	Alleles
Parental	RFLP 1 and 2
Parental	RFLP 3 and 4
Recombinant	RFLP 1 and 4
Recombinant	RFLP 3 and 2

When these two flies mate, the frequency of the four possible progeny can be measured and from this information, the genetic distance between the two RFLP loci (upper and lower) can be determined (Fig. 21.9).

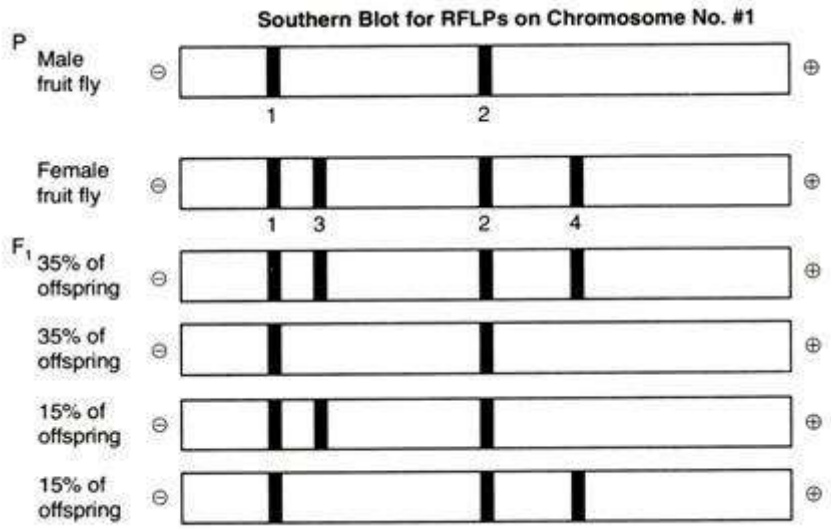


Fig. 21.9. Southern blot for RFLP's on chromosome no. 1.

In this example, 70% of the progeny were produce from parental genotype eggs and 30% were produced by recombinant genotype eggs. Therefore, these two RFLP loci are 30 centiMorgans apart from each other.

iii. PCR-RFLP:

Isolation of sufficient DNA for RFLP analysis is time consuming and labour intensive. However, PCR can be used to amplify very small amounts of DNA, usually in 2-3 hours, to the levels required for RFLP analysis. Therefore, more samples can be analysed in a shorter time. An alternative name for the technique is Cleaved Amplified Polymorphic Sequence (CAPS) assay.

iv. Limitations:

RFLP is a multistep procedure involving restriction enzymatic cleavage, electrophoresis, southern blotting and detection of specific sequences. It is a time consuming process.

Random Amplified Polymorphic DNA (RAPD):

This technique can be used to determine taxonomic identity, assess kinship relationships, detect inter-specific gene flow, analyse hybrid speciation, and create specific probes. Advantages of RAPDs include suitability for work on anonymous genomes, applicability to work where limited DNA is available, efficiency and low expense. It is also useful in distinguishing individuals, cultivars or accessions. RAPDs also have applications in the identification of

asexually reproduced plant varieties for forensic or agricultural purposes, as well as ecological ones.In RAPD by using different primers, molecular characters can be generated that are diagnostic at different taxonomic levels. This is really a stripped-down version of PCR but uses a single sequence in the design of the primer (i.e., two primers are still needed for PCR: the same primer is used at either end).

The primer may be designed specifically, but could be chosen randomly and is used to amplify a series of samples which will include both the material of interest as well as other control samples with which the experimental material needs to be compared. Choice of primer length will be critical to the determination of band complexity in the resulting amplification pattern. Eventually a particular probe will be found that is able to distinguish between the sample of interest and those that are different.

i. Procedure:

Unlike traditional PCR analysis, RAPD (pronounced 'rapid') does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3′ ends of the primers are not facing each other.

Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel (Fig. 21.10). RAPD is an inexpensive yet powerful typing method for many bacterial species

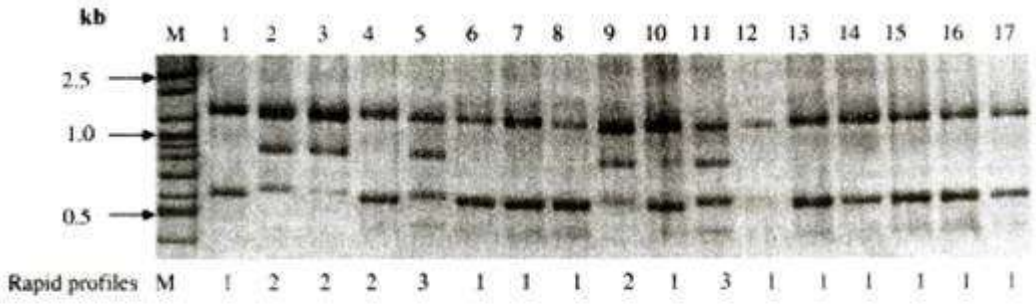


Fig. 21.10. Silver-stained polyacrylamide gel showing three distinct RAPD profiles generated by primer OPE15 for *Haemophilus ducreyi* isolates from different countries. Selecting the right sequence for the primer is very important because different sequences will produce different band patterns and possibly allow for a more specific recognition of individual strains.

RAPD amplification products can be either variable (polymorphic) or constant (non-polymorphic). In a RAPD analysis of several individuals within a species, and species within a genus, constant fragments diagnostic for a genus may be identified, as well as fragments which are polymorphic between species of the genus. RAPDs can be applied to analyse fusion

of genotypes at different taxonomic levels. At the level of the individual, RAPD markers can be applied to parentage analysis, while at the population level, RAPD can detect hybrid populations, species or subspecies.

The detection of genotype hybrids relies on the identification of diagnostic RAPD markers for the parental genotypes under investigation. However RAPD markers tend to underestimate genetic distances between distantly related individuals, for example in inter-specific comparisons. It is wise to be cautious when using RAPD for taxonomic studies above the species level. Conventional RFLP techniques are ill-suited for the analysis of paternity and estimation of reproductive success in species with large offspring clutches, because of the need to determine paternity for each individual offspring. RAPD fingerprinting provides a ready alternative for such cases. Synthetic offspring may be produced by mixing equal amounts of the DNA of the mother and the potential father. The amplification products from the synthetic offspring should ideally contain the full complement of bands that appear in any single offspring of these parents.

ii. Limitations of RAPD:

1. Nearly all RAPD markers are dominant, i.e., it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.
2. PCR is an enzymatic reaction, therefore the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory dependent and needs carefully developed laboratory protocols to be reproducible.
3. Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret.

Amplification Fragment Length Polymorphism (AFLP):

Amplified Fragment Length Polymorphism (AFLP) is a polymerase chain reaction (PCR) based genetic fingerprinting technique that was developed in the early 1990's by Keygene. AFLP can be used in the fingerprinting of genomic DNA of varying origins and complexities. The amplification reaction is rigorous, versatile and robust, and appears to be quantitative.

While AFLP is capable of producing very complex fingerprints (100 bands where RAPD produces 20), it is a technique that requires DNA of reasonable quality and is more experimentally demanding. AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of complementary double stranded adaptors to the ends of the restriction fragments.

A subset of the restriction fragments are then amplified using 2 primers complementary to the adaptor and restriction site fragments. The fragments are visualized on denaturing polyacrylamide gels either through auto-radiographic or fluorescence methodologies.

i. Procedure:

AFLP-PCR is a highly sensitive method for detecting polymorphisms in DNA. The technique was originally described by Vos and Zabeau in 1993. The procedure of this technique is divided into three steps (Fig. 21.11):

1. Digestion of total cellular DNA with one or more restriction enzymes that cuts frequently (Msel, 4 bp recognition sequences) and one that cuts less frequently (EcoRI, 6 bp recognition sequence). The resulting fragments are ligated to end-specific adaptor molecules.
2. Selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences.
3. Electrophoretic separation of amplicons on a gel matrix, followed by visualisation of the band pattern.

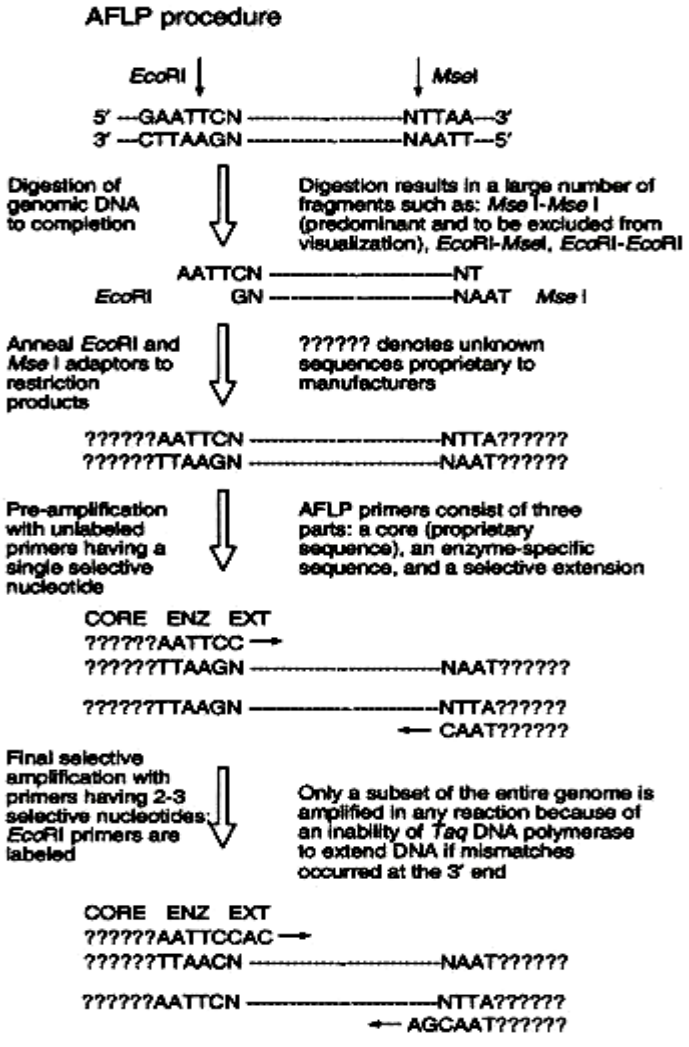


Fig. 21.11. AFLP procedure.

In a second, “selective”, PCR, using the products of the first as template, primers containing two further additional bases, chosen by the user, are used. The EcoRI-adaptor specific primer used bears a label (fluorescent or radioactive). Gel electro-phoretic analysis reveals a pattern (fingerprint) of fragments representing about 1/4000th of the EcoRl-Msel fragments.

AFLP’s, can be co-dominant markers, like RFLP’s. Co-dominance results when the polymorphism is due to sequences within the amplified region. Yet, because of the number of bands seen at one time, additional evidence is needed to establish that a set of bands result from different alleles at the same locus.

If, however, the polymorphism is due to presence/absence of a priming site, the relationship is dominance. The non-priming allele will not be detected as a band. Compared to RAPD, fewer primers should be needed to screen all possible sites. AFLP can be used for mapping, fingerprinting and genetic distance calculation between genotypes. The advantage of AFLP is its high multiplexity and therefore the possibility of generating high marker densities.

One limitation of the AFLP technique is that fingerprints may share few common fragments when genome sequence homology is less than 90%. Therefore, AFLP cannot be used in comparative genomic analysis with hybridization-based probes or when comparing genomes that are evolving rapidly such as those of some microbes. Conversely, very homogeneous genomes may not be suitable for AFLP analysis.

ii. Limitations of AFLP:

1. Proprietary technology is needed to score heterozygotes and homozygotes. Otherwise, AFLP must be dominantly scored.
2. Developing locus-specific markers from individual fragments can be difficult.
3. Need to use different kits adapted to the size of the genome being analysed.

Microsatellites:

Microsatellites can be used to determine genetic diversity within a species, as well as being able to distinguish varieties and even individuals, as well as parentage. The distribution of genetic variability is commonly used to verify species, subspecies or population division. Monitoring change in diversity may also be useful for predicting populations in peril as the persistence of a population partially depends on maintaining its evolutionary significance which requires genetic variation.

Microsatellites have been used to estimate demographic bottlenecks in some species. A bottleneck, when it severely and temporarily reduces population size, can also drastically reduce the genetic diversity of a population. A common theme in conservation genetics is the use of genetic variation to identify populations that have experienced bottlenecks, as numerous

threatened or endangered species and populations have been found to have low levels of genetic variation.

Inter-Simple Sequence Repeats (ISSR):

ISSRs can be used to assess hybridization in natural populations of plants, as a study on Penstemon (Scrophulariaceae) did. Eight ISSR primers were used to examine patterns of hybridization and hybrid speciation in a hybrid complex involving four species, as well as allowing examination of pollen-mediated gene flow. Previous studies using allozymes, restriction-site variation of nuclear rDNA and chloroplast DNA failed to determine whether gene flow occurs among species other than *P. cenranthifolius*.

The previous studies also failed to provide support for hypotheses of diploid hybrid speciation. ISSR proved to be a much more successful technique in this study, allowing all species and all DNA accessions to be differentiated. ISSR has also been used to detect varieties and diversity in rice, revealing much more data than RFLPs. The technique allowed for dissection below the subspecies level and this gives it a good level of applicability in the study of rare or endangered plants.

ISSRs have been used in conjunction with RAPD data to determine the colonization history of Olea europaea in Macronesia, along with lineages in the species complex. The two techniques have also been utilized in examining the historical biogeography of Sea rocket (*Cakile maritima*) and Sea Holly (*Eryngium maritimum*), comparing different and only distantly related taxa of broadly similar extant distribution. The trees generated by the different methods were largely similar topologically. Using the result, dispersal routes of the species along a linear coast line could be construed.

Joint use of RAPD and ISSR has also been used to examine clonal diversity in Calamagrostis *porteri ssp*. insperata (Poaceae), a rare grass that has little or no sexual reproduction, and spreads by vegetative reproduction. The relative advantages and disadvantages of various molecular markers in physical mapping are summarized in Table 21.3. This information suggests that RFLP, SSR and AFLP markers are most effective in detecting polymorphism.

However, given the large amount of DNA required for RFLP detection and the difficulties in automating RFLP analysis, AFLP and SSR are currently most popular markers.

Table 21.3. Comparison of most commonly used marker systems.

Features	RFLP	RAPD	AFLP	SSR	SNP
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	High	High	Moderate	Moderate	High
PCR based	No	Yes	Yes	Yes	Yes
No. of polymorph loci analysed	1.0-3.0	1.5-5.0	20-100	1.0-3.0	1.0
Ease of use	Not easy	Easy	Easy	Easy	Easy
Amenable to automation	Low	Moderate	Moderate	High	High
Reproducibility	High	unreliable	High	High	High
Development cost	Low	Low	Moderate	High	High
Cost per analysis	High	Low	Moderate	Low	Low

The main uses of these markers include:

1. Assessment of genetic variability and characterization of germplasm.
2. Identification and fingerprinting of genotypes.
3. Estimation of genetic distances between population, inbreeds and breeding material.
4. Detection of monogenic and qualitative trait loci.
5. Marker assisted selection.
6. Identification of sequences of useful candidate genes.

B. Restriction Mapping of DNA Fragments:

Restriction Mapping:

Genetic mapping using RFLPs as DNA markers can locate the positions of polymorphic restriction sites within a genome, but very few of the restriction sites in a genome are polymorphic, so many sites are not mapped by this technique.

We increase the marker density on a genome map by using an alternative method to locate the positions of some of the non polymorphic restriction sites. This is what restriction mapping achieves, although in practice the technique has limitations that means it is applicable only to relatively small DNA molecules.

Methodology for Restriction Mapping:

The simplest way to construct a restriction map is to compare the fragment sizes produced when a DNA molecule is digested with two different restriction enzymes that recognize different target sequences. An example using the restriction enzymes EcoRI and BamHI is shown in figure. 21.12. First, the DNA molecule is digested with just one of the enzymes and the sizes of the resulting fragments measured by agarose gel electrophoresis. Next, the molecule is digested with the second enzyme and the resulting fragments again sized in an agarose gel.

The results of subsequent use of two enzymes give clear picture about restriction sites creating a large number of fragments but this method do not allow their relative positions to be determined. Additional information is therefore obtained by cutting the DNA molecule with both enzymes together. In the example shown in Figure 21.12, the double restriction enables three of the sites to be mapped. However, a problem arises with the larger EcoRI fragment because this contains two BamHI sites and there are two alternative possibilities for the map location of the outer one of these.

The problem dissolved by going back to the original DNA molecule and treating it again with BamHI on its own, but this time preventing the digestion from going to completion by, for example, incubating the reaction for only a short time rousing a suboptimal incubation temperature. This is called a partial restriction and leads to a more complex set of products. The complete restriction products now being supplemented with partially restricted fragments that still contain one or more uncut BamHI sites.

In the example shown in Figure 21.12, the size of one of the partial restriction fragments is diagnostic and the correct map can be identified. A partial restriction usually gives the information needed to complete a map, but if there are many restriction sites then this type of analysis becomes bulky, simply because there are many different fragments to consider. An alternative strategy is simpler because it enables the majority of the fragments to be ignored. This is achieved by attaching a radioactive or other type of marker to each end of the starting DNA molecule before carrying out the partial digestion.

The result is that many of the partial restriction products become “invisible” because they do not contain an end-fragment and so do not show up when the agarose gel is screened for labeled products (Fig. 21.12). The sizes of the partial restriction products that are visible enable unmapped sites to be positioned relative to the ends of the starting molecule.

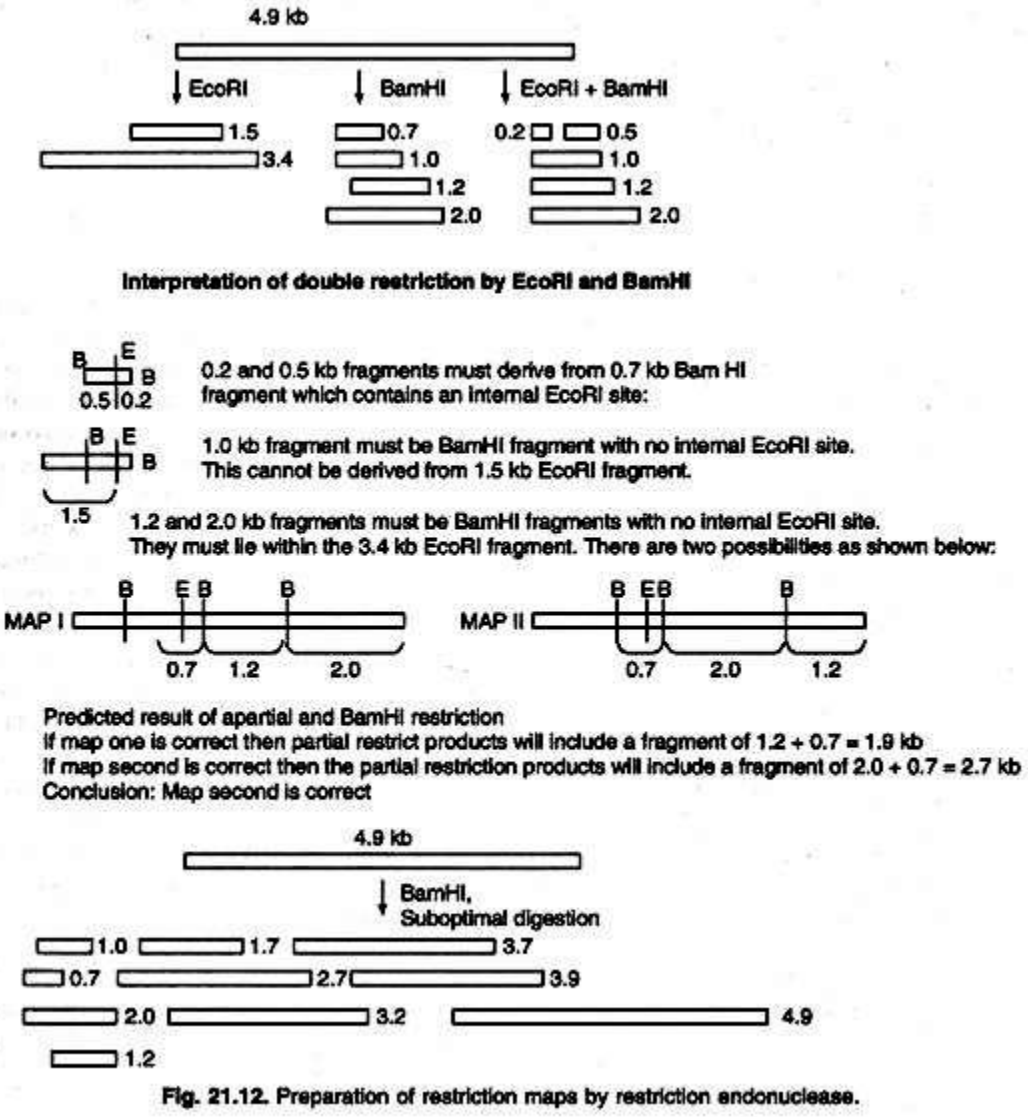


Fig. 21.12. Preparation of restriction maps by restriction endonuclease.

The scale of restriction mapping is limited by the sizes of the restriction fragments. Restriction maps are easy to generate if there are relatively few cut sites for the enzymes being used. However, as the number of cut sites increases, so also do the numbers of single, double and partial-restriction products whose sizes must be determined and compared in order for the map to be constructed. Computer analysis can be brought into play but problems still eventually arise.

A stage will be reached when a digest contains so many fragments that individual bands merge on the agarose gel, increasing the chances of one or more fragments being measured incorrectly or missed out entirely. If several fragments have similar sizes then even if they can all be identified, it may not be possible to assemble them into a clear map. Restriction mapping is therefore more applicable to small rather than large molecules, with the upper limit for the technique depending on the frequency of the restriction sites in the molecule being mapped.

In practice, if a DNA molecule is less than 50 kb in length it is usually possible to construct a clear restriction map for a selection of enzymes with six nucleotide recognition sequences. Restriction maps are equally useful after bacterial or eukaryotic genomic DNA has been cloned, if the cloned fragments are less than 50kb in length, because a detailed restriction map can then be built up as a preliminary to sequencing the cloned region. This is an important application of restriction mapping in projects sequencing large genomes.
Restriction mapping can be used for mapping of entire genomes larger than 50kb by slightly eliminating the limitations of restriction mapping by choosing enzymes expected to have infrequent cut sites in the target DNA molecule.

These "rare cutters" fall into two categories:
1. A few restriction enzymes cut at seven- or eight-nucleotide recognition sequences. Examples are SapI (5′-GCTCTTC-3′) and SgfI (5′-GCGATCGC-3′). The enzymes with seven-nucleotide recognition sequences would be expected, on average, to cut a DNA molecule with GC content of 50% once every 47 = 16,384 bp.
The enzymes with eight nucleotide recognition sequences should cut once every 48 = 65,536 bp. These figures compare with 46 = 4096 bp for enzymes with six-nucleotide recognition sequences, such as BamHI and EcoRI. Cutters with seven-or eight-nucleotide recognition sequences are often used in restriction mapping of large molecules, but the approach is not as useful as it might by simply because not many of these enzymes are known.

2. Enzymes can be used whose recognition sequences contain motifs that are rare in the target DNA. Genomic DNA molecules do not have random sequences and some molecules are significantly deficient in certain motifs. For example, the sequence 5′-CG- 3′ is rare in the genomes of vertebrates because vertebrate cells possess an enzyme that adds a methyl group to carbon 5 of the C nucleotide in this sequence. Domination of the resulting 5-methylcytosine gives thymine. The consequence is that during vertebrate evolution, many of the 5′-CG'3 sequences that were originally in these genomes have become converted to 5′-TG-3′.
Restriction enzymes that recognize a site containing 5′- CG-3′ therefore cut vertebrate DNA relatively infrequently. Examples are SmaI (5′-CCCGGG-3′), which cuts human DNA once every 78 kb on average, and BssHII (5′- GCGCGC'3′), which cuts once every 390 kb. The potential of restriction mapping is therefore increased by using rare cutters. It is still not possible to construct restriction maps of the genomes of animals and plants, but it is feasible to use the technique with large cloned fragments, and with the smaller DNA molecules of prokaryotes and lower eukaryotes such as yeast and fungi.
If a rare cutter is used then it may be necessary to employ a special type of agarose gel electrophoresis to study the resulting restriction fragments. This is because the relationship between the length of DNA molecule and its migration rate in an electrophoresis gel is not

linear, the resolution decreasing as the molecules get longer (Fig. 21.13A). This means that it is not possible to separate molecules more than about 50 kb in length, because all of these longer molecules run as a single, slowly migrating band in a standard agarose gel.

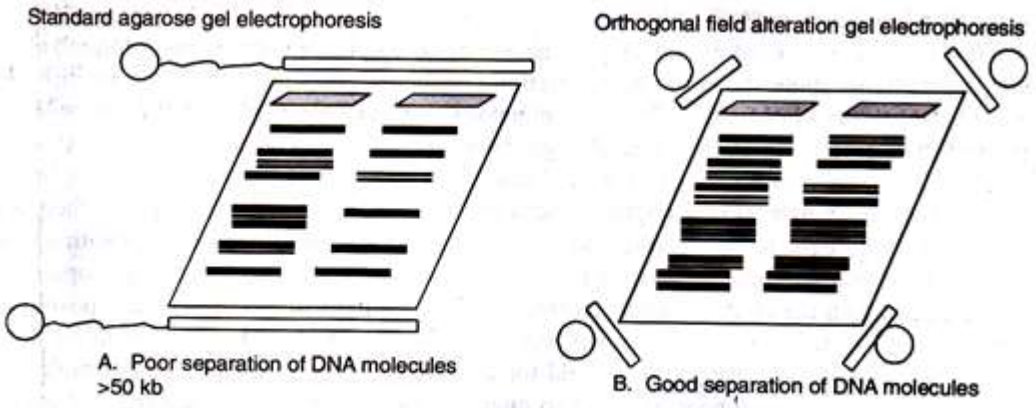


Fig. 21.13. A,B Conventional and non-conventional agarose gel electrophoresis.

To separate them it is necessary to replace the linear electric field used in conventional gel electrophoresis with a more complex field. An example is provided by orthogonal field alternation gel electrophoresis (OFAGE), in which the electric field alternates between two pairs of electrodes, each positioned at an angle of 45″ to the length of the gel (Fig. 21.13B).
The DNA molecules still move down through the gel, but each change in the field forces the molecules to realign. Shorter molecules realign more quickly than longer ones and so migrate more rapidly through the gel. The overall result is that molecules much longer than those separated by conventional gel electrophoresis can be resolved. Related techniques include CHEF (contour clamped homogeneous electric fields) and FIGE (field inversion gel electrophoresis).

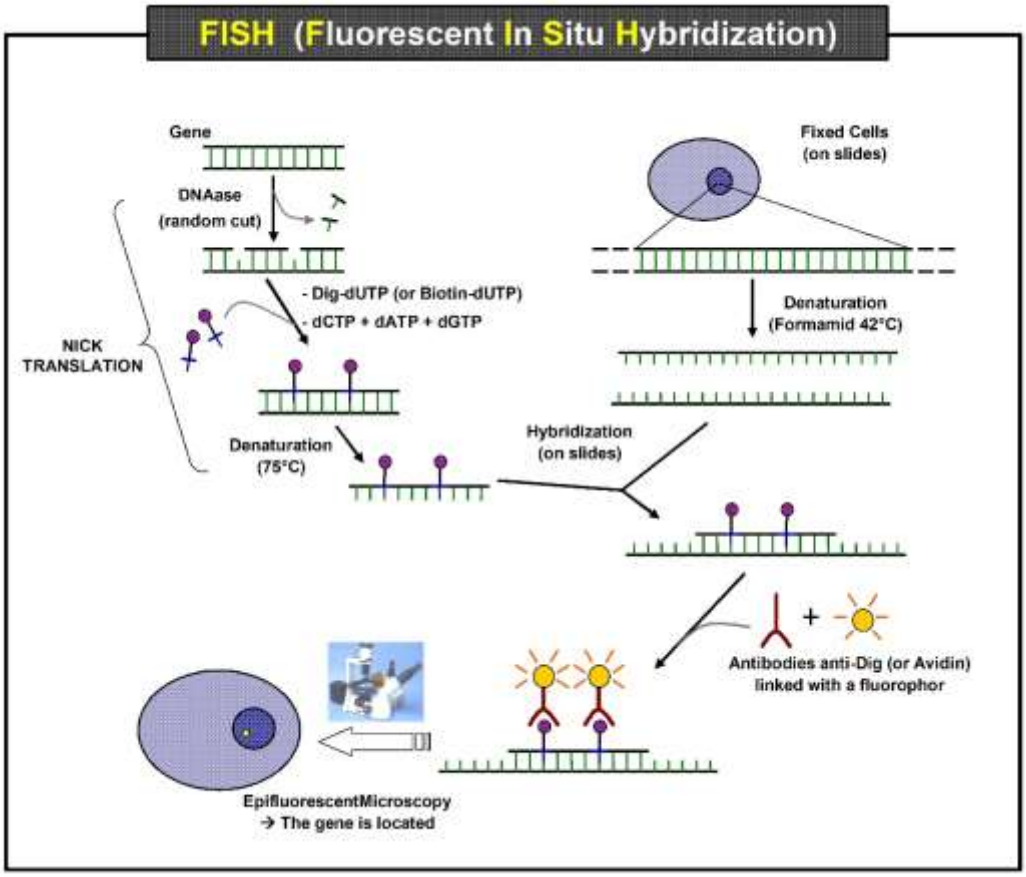
Fluorescent In Situ Hybridization (FISH):

FISH (Fluorescent in situ hybridization) is a cytogenetic technique which can be used to detect and localize the presence or absence of specific DNA sequences on chromosomes. It uses fluorescent probes which bind only to those parts of the chromosome with which they show a high degree of sequence similarity. FISH has evolved into a core technique spanning many areas of diagnosis and research, including cancer cytogenetics, prenatal screening of chromosomal aberrations, molecular pathology and developmental molecular biology. The general principle of FISH, hybridizing fluorescently-labelled loci-specific, telomeric or centromeric DNA probes to chromosomes, can be applied to a range of specimens such as metaphase chromosome spreads or to inter-phase nuclei and cells.

Fluorescence microscopy can be used to find out where the fluorescent probe bound to the chromosome. FISH is often used for finding specific features in DNA. These features can be used in genetic counselling, medicine, and species identification. First, a probe is constructed. The probe has to be long enough to hybridize specifically to its target (and not to similar sequences in the genome), but not too large to impede the hybridization process, and it should be tagged directly with fluorophores, with targets for antibodies or with biotin. This can be done in various ways, for example nick translation and PCR using tagged nucleotides.

Then, an inter-phase or metaphase chromosome preparation is produced. The chromosomes are firmly attached to a substrate, usually glass slides. Repetitive DNA sequences must be blocked by adding short fragments of DNA to the sample. The probe is then applied to the chromosome DNA and incubated for ~12 hours while hybridizing. Several wash steps remove all un-hybridized or partially hybridized probes. The results are then visualized and quantified using a microscope that is capable of exciting the dye and recording images. If the fluorescent signal is weak, amplification of the signal may be necessary in order to exceed the detection threshold of the microscope. The signal strength depends on many factors; probe labelling efficiency, the type of probe and the type of dye affect the fluorescent signal. Fluorescently-tagged antibodies or streptavidin are bound to the dye molecule. These secondary components are selected so that they have a strong signal. The technique of in situ hybridization is widely used to cytogenetically map the locations of genes or other DNA sequences within large eukaryotic chromosomes. The term *in situ* (from the Latin for “in place”) indicates that the procedure is conducted on chromosomes that are being held in place-adhered to a surface.

To map a gene via in situ hybridization, researchers use a probe to detect the location of the gene within a set of chromosomes. If the gene of interest has been cloned previously, as described in Chapter 18 , the DNA of the cloned gene can be used as a probe. Because a DNA strand from a cloned gene, which is a very small piece of DNA relative to a chromosome, hybridizes only to its complementary sequence on a particular chromosome, this technique provides the ability to localize the gene of interest. For example, let’s consider the gene that causes the white-eye phenotype in *Drosophila* when it carries a loss-of-function mutation. This gene has already been cloned. If a single-stranded piece of this cloned DNA is mixed with *Drosophila* chromosomes in which the DNA has been denatured, it will bind only to the X chromosome at the location corresponding to the site of the eye colour gene.



The most common method of in situ hybridization uses fluorescently labelled DNA probes and is referred to as fluorescence in situ hybridization (FISH)**.**

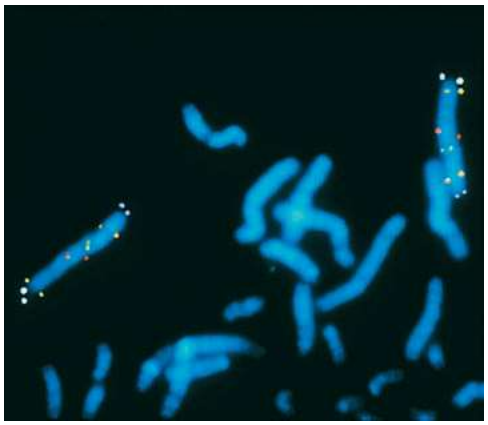


Figure : The results of FISH

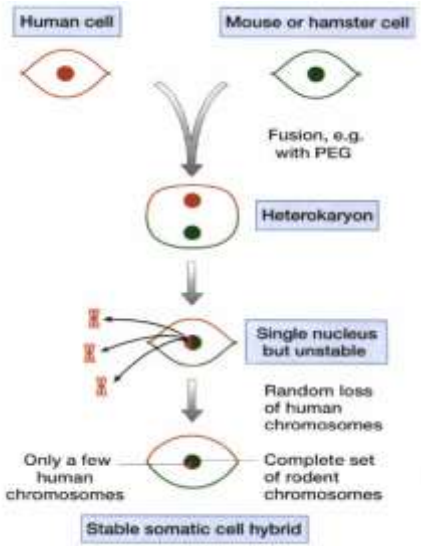
The cells are prepared using a technique that keeps the chromosomes intact. The cells are treated with agents that cause them to swell, and their contents are fixed to the slide. The chromosomal DNA is then denatured, and a DNA probe is added. For example, the added DNA probe might be single-stranded DNA that is complementary to a specific gene. In this case, the goal of a FISH experiment is to determine the location of the gene within a set of chromosomes. The probe binds to a site in the chromosomes where the gene is located because the probe and

chromosomal gene line up and hydrogen bond with each other. To detect where the probe has bound to a chromosome, the probe is subsequently tagged with a fluorescent molecule. This is usually accomplished by first incorporating biotin-labelled nucleotides into the probe. Biotin, a small, nonfluorescent molecule, has a very high affinity for a protein called avidin. Fluorescently labelled avidin is added, which binds tightly to the biotin and thereby labels the probe as well.

Radiation Hybrid Mapping:

Radiation hybrid (RH) mapping is the most efficient method for generating long range genomic maps using both polymorphic and non-polymorphic markers. There are a number of advantages to radiation hybrid mapping. Non-polymorphic markers are informative, making them particularly useful for anchoring clone contigs which frequently do not contain polymorphic markers. RHs offer high map resolution for relatively small numbers of hybrids. A high-resolution (100 kb) whole genome map can be generated using fewer than 100 hybrids. Apart from the efficiency of RH mapping as a stand-alone mapping tool, the additional strength of this technique lies in allowing the efficient integration of physical and genetic maps, by facilitating the resolution of co-segregating genetic markers lying in recombination cool-spots on the meiotic map, and anchoring Yeast Artificial Chromosome (YAC) contigs to the genome map.

In radiation hybrid mapping, human chromosomes are separated from one another and broken into several fragments using high doses of X rays. Similar to the underlying principle of mapping genes by linkage analysis based on recombination events, the farther apart two DNA markers are on a chromosome, the more likely a given dose of X rays will break the chromosome between them and thus place the two markers on two different chromosomal fragments. The order of markers on a chromosome can be determined by estimating the frequency of breakage that, in turn, depends on the distance between the markers. This technique has been used to construct whole-genome radiation hybrid maps.



Technique :

A rodent-human somatic cell hybrid ("artificial" cells with both rodent and human genetic material), which contains a single copy of the human chromosome of interest, is X-irradiated. This breaks the chromosome into several pieces, which are subsequently integrated into the rodent chromosomes. In addition, the dosage of radiation is sufficient to kill the somatic cell hybrid or donor cells, which are then rescued by fusing them with nonirradiated rodent recipient cells. The latter, however, lack an important enzyme and are also killed when grown in a specific medium. Therefore, the only cells that can survive the procedure are donor-recipient hybrids that have acquired a rodent gene for the essential enzyme from the irradiated rodent-human cell line.

From these donor-recipient hybrids, clones can be isolated and tested for the presence or absence of DNA markers on the human chromosome of interest, and the frequencies with which markers were retained in each clone can be calculated. This process is complicated by the fact that hybrids may contain more than one DNA fragment. For example, two markers retained in one hybrid may result from retention of the two markers on separate fragments or from no break between the markers. However, the frequency of breakage, theta, can be estimated using statistical methods, and a lod score (logarithm of the likelihood ratio for linkage) can be calculated to identify significantly linked marker pairs.

The basic principle of RH mapping is that the closer two loci are on a chromosome, the less likely they are to be separated by a radiation-induced break. Therefore, markers that are closely linked show correlated co-retention patterns across the hybrid panel, where markers located a large distance from one another are retained almost independently. WG-RH mapping has been reviewed extensively elsewhere. The two most extensively used RH mapping programs are RHMAP and RHMAPPER. In this chapter we will demonstrate the analysis of two WG-RH datasets using these programs, discussing the merits and limitations of both packages.

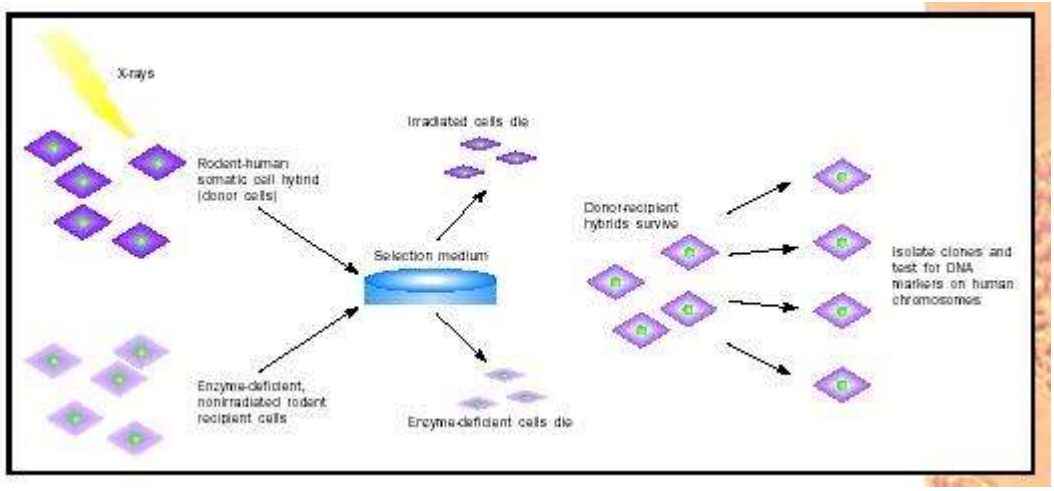


Figure: Radiation hybrid mapping process.

Genome sequencing:

When sequencing an entire genome, researchers must consider factors such as genome size, the efficiency of the methods used to sequence DNA, and the costs of the project. Since genome sequencing projects began in the 1990s, researchers have learned that the most efficient and inexpensive way to sequence genomes is via an approach called shotgun sequencing, in which

DNA fragments to be sequenced are randomly generated from larger DNA fragments. In this method, genomic DNA is isolated and broken into smaller DNA fragments, typically 1500 bp or longer in length. Until recently, the researchers then used the technique of dideoxy sequencing, to randomly sequence fragments from the genome. As a matter of chance, some of the fragments overlap**.** The DNA sequences in two different fragments are identical in the overlapping region. This allows researchers to order them as they are found in the intact chromosome.

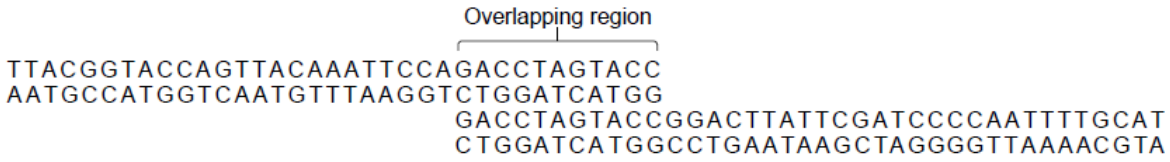


Fig 6: Overlapping sequence of DNA comparison

An advantage of shotgun DNA sequencing is that it does not require extensive mapping, which can be very time-consuming and expensive. A disadvantage is that researchers waste some time sequencing the same region of DNA more times than needed. For many genome-sequencing projects in the past, researchers typically followed two types of shotgun strategies: hierarchical shotgun sequencing or whole-genome shotgun sequencing.

Shotgun sequencing strategies: In the late 1990s, when researchers first began to sequence the larger genomes from eukaryotic species, they first took the approach of hierarchical shotgun sequencing. To begin this process, researchers cloned large (e.g., 150,000 bp long) DNA fragments into BACs (or YACs) to generate a contig for each chromosome. For DNA sequencing, researchers chose a set of clones from each chromosome contig that had relatively short overlapping regions. Each BAC clone in a chosen set was then subjected to shotgun sequencing. A clone was broken into smaller pieces (e.g., 2000 bp), and those small pieces were cloned into vectors. The recombinant vectors were then randomly sequenced using a primer that annealed at a site next to the inserted DNA. The method of dideoxy sequencing yielded a DNA sequence, usually 500 to 1000 bp in length, at one end of each insert. The resulting DNA sequences were analysed by a computer program that identified overlapping sequences between two DNA fragments and assembled the DNA sequence into one long sequence along a single chromosome.

In the hierarchical method, the nucleotide sequence was determined clone by clone. For this reason, the method is also called the clone-by-clone or BAC-to-BAC approach. This strategy had the advantage of making it easier to align the overlapping sequences because the DNA sequences were grouped according to the BAC clone from which the sequence was obtained.

Another issue is that this method minimized the amount of DNA sequencing by reducing the amount of sequencing in overlapping regions. This was an advantage in the 1990s when DNA sequencing was an expensive and labour-intensive procedure. However, a disadvantage of this approach is that BAC mapping can be time consuming and expensive. In more recent years, advances in DNA sequencing technology greatly diminished the time and expense of dideoxy sequencing. Genome-sequencing projects in the 1990s through the first decade of the 21st century more commonly followed a strategy called whole-genome shotgun sequencing**.** In this

method, the physical mapping step used in the hierarchical approach was bypassed. The DNA from the entire genome was isolated and sheared into small (e.g., 2000- or 10,000-bp) and large (50,000- or 150,000-bp) fragments and cloned into vectors. Each insert was usually sequenced at both ends-an approach called double-barrel shotgun sequencing. As in the hierarchical approach, the DNA sequences, which were 500 bp to 1000 bp in length, were then analysed by a computer program that identified overlapping regions between two DNA fragments and assembled the data into one long sequence.

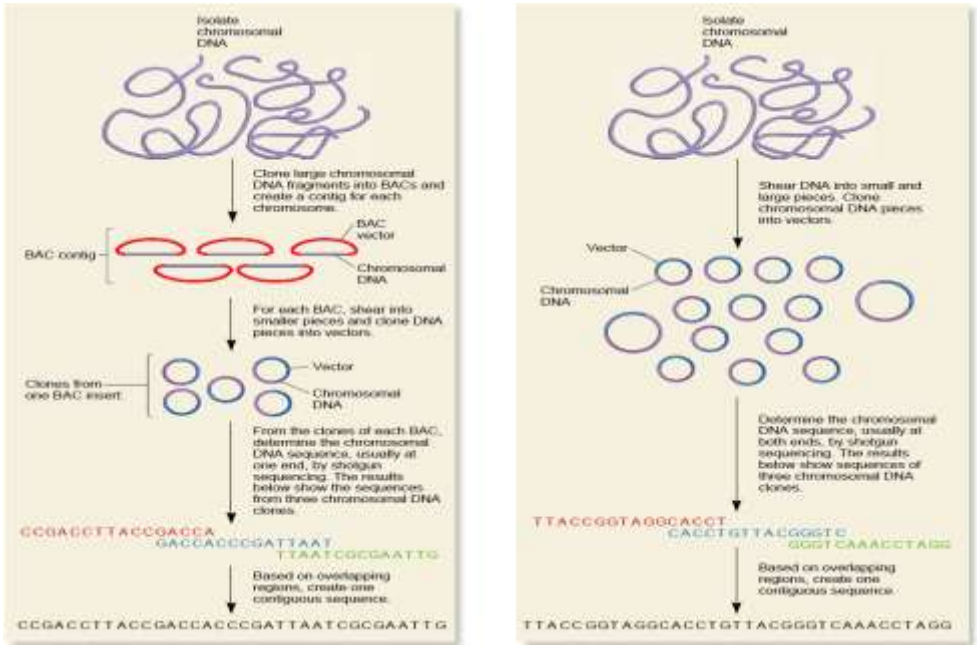


Fig 5: Two approaches of genome shotgun sequencing - Hierarchical genome shotgun (left) and Whole genome shotgun (right)

The double-barrel strategy improved the ability of researchers to align their DNA sequences. Most of the DNA sequencing in a whole genome shotgun approach was carried out on small DNA fragments. However, due to the prevalence of repetitive sequences, particularly within the genomes of eukaryotic species, the alignment of small pieces sometimes was difficult. The sequencing of both ends of longer fragments made alignment easier, because the relative spacing of particular DNA sequences was known. Both hierarchical and whole genome shotgun sequencing involve the cloning of DNA into vectors. Newer methods of DNA sequencing no longer require this cloning step.

DNA Sequencing Definition

DNA sequencing is the process of determining the sequence of nucleotides within a DNA molecule. Every organism's DNA consists of a unique sequence of nucleotides. Determining the sequence can help scientists compare DNA between organisms, which can help show how the organisms are related.

DNA Sequencing Overview

This means that by sequencing a stretch of DNA, it will be possible to know the order in which the four nucleotide bases – adenine, guanine, cytosine, and thymine – occur within that nucleic acid molecule. The necessity of DNA sequencing was first made obvious by Francis Crick's theory that the sequence of nucleotides within a DNA molecule directly influenced the amino acid sequences of proteins. At the time, the belief was that a completely sequenced genome would lead to a quantum leap in understanding the biochemistry of cells and organisms. **Modern DNA sequencing consists of high-throughput methods which allow entire DNA sequences to be discovered in a matter of hours.** This technology has allowed many companies to start offering at-home DNA testing. Many of the "results" found by these tests are simply correlations found between a genetic variant and a certain condition. However, technology has also allowed scientists to test the DNA of many organisms to better understand evolutionary relationships.

DNA Sequencing Example

Though DNA sequencing used to take years, it can now be done in hours. **Further, the first full sequence of human DNA took around 3 billion dollars.** Now, certain companies will sequence your entire genome for less than $1,000. The most advanced tests will analyze every nucleotide within your genome. However, many companies now offer single-nucleotide polymorphism tests.

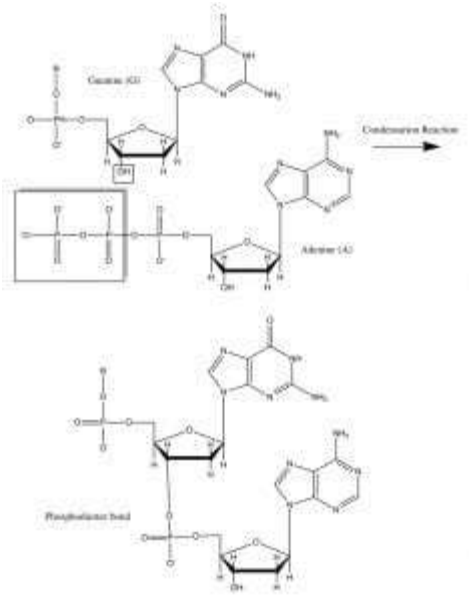
These tests focus on individual nucleotides within genes that can signify certain genetic variants. **These SNPs, as they are known, have been correlated to certain conditions and can help predict how your genes may influence your life.** Some SNPs are related to various diseases, while others are related to your metabolism and how your body processes nutrients. Thousands of different correlations have been found, and DNA sequencing can be used to figure out how your genome affects your life.

DNA Sequencing Methods

There are two main types of DNA sequencing. **The older, classical chain termination method is also called the Sanger method.** Newer methods that can process a large number of DNA molecules quickly are collectively called High-Throughput Sequencing (HTS) techniques or Next-Generation Sequencing (NGS) methods.

Sanger Sequencing

The Sanger method relies on a **primer that binds to a denatured DNA molecule** and initiates the synthesis of a single-stranded polynucleotide in the presence of a DNA polymerase enzyme, using the denatured DNA as a template. In most circumstances, the enzyme catalyses the addition of a nucleotide. A covalent bond, therefore, forms between the 3′ carbon atom of the deoxyribose sugar molecule in one nucleotide and the 5′ carbon atom of the next. This image below shows how this bond is formed.



A sequencing reaction mixture, however, would have a small proportion of modified nucleotides that cannot form this covalent bond due to the absence of a reactive hydroxyl group, giving rise to the term ‘dideoxyribonucleotides’, i.e., they do not have a 2’ or 3’ oxygen atom when compared to the corresponding ribonucleotide. This would terminate the DNA polymerization reaction prematurely. At the end of multiple rounds of such polymerizations, a mixture of molecules of varying lengths would be created.

In the earliest attempts at using the Sanger method, the DNA molecule was first amplified using a labelled primer and then split into four test tubes, each having only one type of ddNTP. That is, each reaction mixture would have only one type of modified nucleotide that could cause chain termination. After the four reactions were completed, the mixture of DNA molecules created by chain termination would undergo electrophoresis on a polyacrylamide gel, and get separated according to their length.

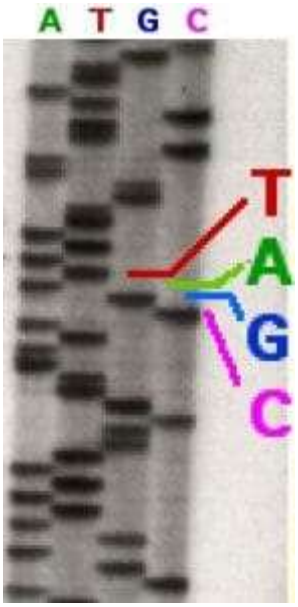


Figure: In the image above, a sequencing reaction with ddATP was electrophoresed through the first column. Each line represents a DNA molecule of a particular length, the result of a polymerization reaction that was terminated by the addition of a ddATP nucleotide. The second, third and fourth columns contained ddTTP, ddGTP, and ddCTP respectively.

With time, this method was modified so that each ddNTP had a different fluorescent label. The primer was no longer the source of the radiolabel or fluorescent tag. Also known as dye-terminator sequencing, this method used four dyes with non-overlapping emission spectra, one for each ddNTP.

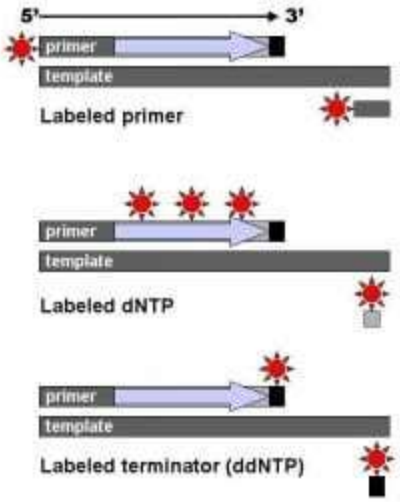


Figure: The image shows the difference between labeled primers, labeled dNTPs and dyed terminator NTPs.

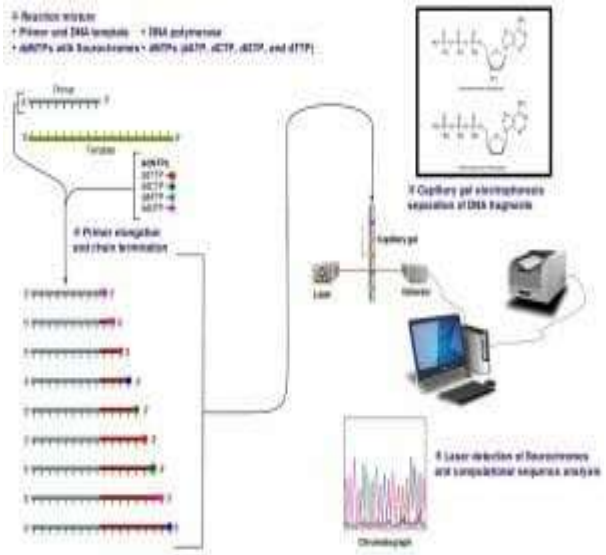


Figure: The image above shows a schematic representation of dye-terminator sequencing. There is a single reaction mixture carrying all the elements needed for DNA elongation. The reaction mixture also contains small concentrations of four ddNTPs, each with a different fluorescent tag. The completed reaction is run on a capillary gel. The results are obtained through an analysis of the emission spectra from each DNA band on the gel. A software program then analyses the spectra and presents the sequence of the DNA molecule.

High Throughput Sequencing

Sanger sequencing continues to be useful for determining the sequences of relatively long stretches of DNA, especially at low volumes. **However, it can become expensive and laborious when a large number of molecules need to be sequenced quickly.** Ironically, though the traditional dye-terminator method is useful when the DNA molecule is longer, high-throughput methods have become more widely used, especially when entire genomes need to be sequenced.

There are three major changes compared to the Sanger method. The first was the development of a cell-free system for cloning DNA fragments. Traditionally, the stretch of DNA that needed to be sequenced was first cloned into a prokaryotic plasmid and amplified within bacteria before being extracted and purified. High throughput sequencing or next-generation sequencing technologies no longer relied on this labour-intensive and time-intensive procedure. Secondly, these methods created space to run millions of sequencing reactions in parallel. **This was a huge step forward from the initial methods where eight different reaction mixtures were needed to produce a single reliable nucleotide sequence.** Finally, there is no separation between the elongation and detection steps. The bases are identified as the sequencing reaction proceeds. While HTS decreased cost and time, their 'reads' were relatively short. That is, in order to assemble an entire genome, intense computation is necessary. The advent of HTS has vastly expanded the applications for genomics. DNA sequencing has now become an integral part of basic science, translational research, medical diagnostics, and forensics.

Next generation sequencing: The ability to rapidly sequence large amounts of DNA is often referred to as high-throughput sequencing . Different types of technological advances have made this possible. First, different aspects of DNA sequencing have become automated so that samples can be processed rapidly in a machine. It is understood that fluorescently labelled nucleotides can automate the ability to read a DNA sequence by using a fluorescence detector. A second advance from high- throughput sequencing technologies is they enable parallel sequencing. This means multiple samples can be processed at once. The first sequencing machines, also called platforms, could simultaneously perform many sequencing runs via multiple capillary gels. For example, DNA-sequencing machines produced by Applied Biosystems, which rely on Sanger dideoxy sequencing, run 96 capillaries in parallel. Each

capillary gel is capable of producing between 500 and 1000 bases of DNA sequence. Although the Sanger dideoxy sequencing method is still in use, newer high-throughput platforms are based on different methods of sequencing DNA. Some of which are often referred to as next-generation sequencing technologies because they have superseded the Sanger dideoxy method for large sequencing projects.

One key technological advance is the ability to process thousands or even millions of sequence reads in parallel rather than only 96 at a time. This massively parallel throughput may require only one or two instrument runs to complete the sequencing of an entire prokaryotic genome. Also, next- generation sequencers are able to use samples that contain

mixtures of DNA fragments that have not been subjected to the conventional vector-based cloning. The elimination of such cloning steps saves a great deal of time and money.

Second-Generation DNA Sequencers			
Technology*	**DNA Preparation**	**Enzyme(s) Used**	**Detection**
Roche 454/FLX Pyrosequencer	DNA fragments are bound to small beads, which are dropped into tiny wells in a fiber optic chip.	DNA polymerase, ATP sulfurylase, luciferase, apyrase	Pyrophosphate release activates luciferase, which gives off light.
Illumina/Solexa Genome Analyzer	DNA fragments are bound to a flow cell surface.	DNA polymerase	Four different fluorescently labeled nucleotides are detected.
Third-Generation DNA Sequencers			
Pacific Biosciences SMRT	DNA fragments and DNA polymerase are trapped within tiny holes on a thin metal film.	DNA polymerase	The growth of individual DNA molecules is monitored by fluorescence imaging
Helicos Biosciences tSMS	DNA fragments are bound to a flow cell surface.	DNA polymerase	The growth of individual DNA molecules is monitored by fluorescence imaging.
ZS Genetics TEM	DNA is labeled with heavier elements, such as iodine or bromine.	None	The DNA sequence of a single molecule is read via transmission electron microscopy (TEM).

Table : Example of Next-generation sequencing technology

The newer sequencing platforms employ a complex interplay of enzymology, chemistry, high-resolution optics, and new approaches to processing the data. These instruments allow for easy sample preparation steps prior to DNA sequencing. Most of them involve strategies in which fragmented DNA is immobilized in a fixed position and repeatedly exposed to reagents. The second-generation sequencing platforms use PCR to amplify the DNA, whereas third-generation sequencers actually read single DNA molecules. As an example of next-generation sequencing, considers the technology called pyrosequencing, which was developed by Pal Nyren and Mostafa Ronaghi in 1996 and is the basis for the Roche 454/FLX Pyrosequencer. Samples, such asgenomic DNA, are broken into small 300- to 800-bp fragments. Short oligonucleotides called adaptors are then linked to the 5′and 3′ ends of the DNA fragments. The DNA is then denatured into single strands. The mixture of single-stranded DNA fragment

with adaptors is called the sample library. The single-stranded DNA fragments are then attached to beads via the adaptors. Initially, just one DNA strand is attached per bead. The beads are emulsified in an oil–water mixture such that each bead becomes localized into a single droplet. The mixture also contains PCR reagents, including primers that are complementary to the adaptors. During this step, the single-stranded DNA on the bead becomes amplified into many identical products, which also become attached to the beads via the adaptors. At this stage, each bead contains about one million copies of a particular DNA segment. Next, each bead is placed into a well of a picotiter plate; the diameter of each well is large enough to accommodate only one bead. Sequencing reagents, which include primers that are complementary to the adaptors, are added to the wells. The picotiter plate acts like a flow cell, which allows pure solutions of nucleotides to be added in a stepwise fashion. The synthesis of DNA is monitored in real time. Therefore, this type of method is referred to as sequencing by synthesis (SBS) because it involves the identification of each nucleotide immediately after its incorporatio into a DNA strand by DNA polymerase.

When a solution has been added that contains one particular nucleotide, how does the machine determine if that nucleotide has been added to a growing DNA strand? The pyrosequencing method relies on the functions of other enzymes, ATP sulfurylase, luciferase, and apyrase, along with additional molecules called adenosine 5′ phosphosulfate and luciferin, which are also included in the sequencing reaction. If a nucleotide has been incorporated into a DNA strand, pyrophosphate is released. ATP sulfurylase uses that pyrophosphate along with adenosine 5′ phosphosulfate to make ATP. The ATP is then used by luciferase to break down luciferin. This reaction gives off light, which is detected by a camera in the sequencing machine. Therefore, light is given off only when a nucleotide is incorporated into a DNA strand. Unincorporated nucleotides and ATP are degraded by apyrase, and the reaction can start again by flowing a new solution containing a particular nucleotide over the picotiter plate. By sequentially adding solutions with only one of the four possible nucleotides (A, T, G, or C), the sequence of the DNA strand can monitored in real time by determining when light is given off.

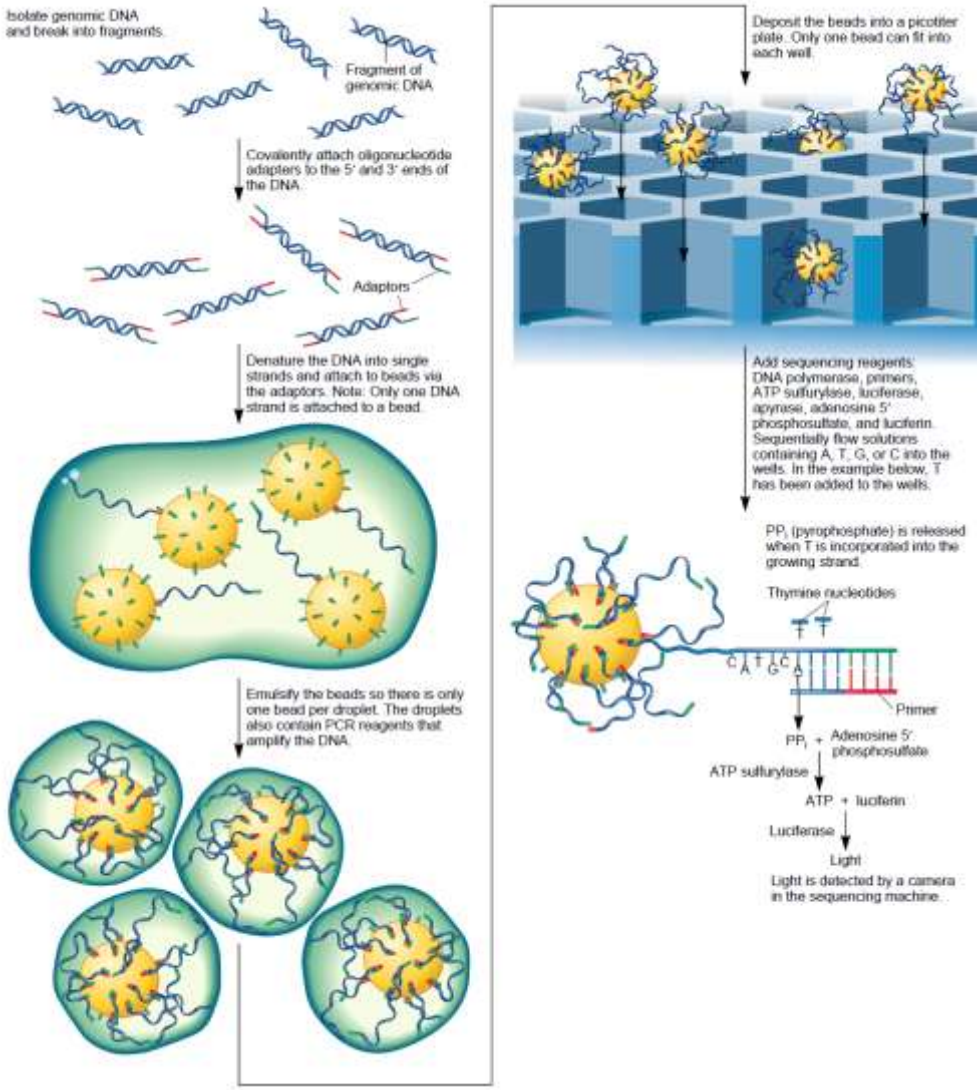


Figure: Pyrosequencing, a new high throughput next generation sequencing technology

Uses of DNA Sequencing:

Traditional, chain-termination technology and HTS methods are used for different applications today. Sanger sequencing is now used mostly for ***de novo*** initial sequencing of a DNA molecule to obtain the primary sequence data for an organism or **gene**. The relatively short 'reads' coming off an HTS reaction (30-400 base pairs compared to the nearly a thousand base pair 'reads' from Sanger sequencing methods) make it difficult to create the entire genome of an organism from HTS methods alone. Occasionally, Sanger sequencing is also needed to validate the results of HTS.

On the other hand, HTS allows the use of DNA sequencing to understand single-nucleotide polymorphisms – among the most common types of **genetic variation** within a **population**. This becomes important in evolutionary biology as well as in the detection of mutated genes that can result in disease. For instance, sequence variations in samples from lung adenocarcinoma allowed the detection of rare mutations associated with the disease. The

chromatin binding sites for specific nuclear proteins can also be accurately identified using these methods. Overall, DNA sequencing is becoming an integral part of many different applications.

a. Diagnostics:

Genome sequencing is particularly useful for identifying the causes of rare genetic disorders. **While more than 7800 diseases are associated with a Mendelian inheritance pattern, less than 4000 of those diseases have been definitively linked to a specific gene or** mutation**.** Early analysis of the exon-genome, or exome, consisting of all the expressed genes of an organism, showed promise in identifying the causal alleles for many inherited illnesses. In one particular case, sequencing the genome of a child suffering from a severe form of inflammatory bowel disease connected the illness to a mutation in a gene associated with inflammation – XIAP. While the patient initially showed multiple symptoms suggestive of an immune deficiency, a bone marrow transplant was recommended based on the results of DNA sequencing. The child subsequently recovered from the ailment.
In addition, HTS has been an important player in developing a greater understanding of tumours and cancers. Understanding the genetic basis of a tumour or cancer enables doctors to have an extra tool in their kit for making diagnostic decisions. **The Cancer Genome Atlas and International Cancer Genome Consortium have sequenced a large number of tumour and demonstrated that these growths can vary vastly in terms of their mutational landscape.** This has also given a better understanding of the kind of treatment options that are ideal for each patient. For instance, the sequencing of the breast cancer genome identified two genes – BRCA1 and BRCA2 – whose pathogenic variants have an enormous impact on the likelihood of developing breast cancer. People with some pathogenic alleles even choose to have preventive surgeries such as double mastectomies.

b. Molecular Biology:

DNA sequencing is now an integral part of most biological laboratories. It is used to verify the results of cloning exercises to understand the effect of particular genes. **HTS technologies are used to study variations in the genetic compositions of plasmids, bacteria, yeast, nematodes or even mammals used in laboratory experiments.** For instance, a cell line derived from breast cancer tissue, called HeLa, is used in many laboratories around the world and was earlier considered as a reliable cell line representing human breast tissue. Recent sequencing results have demonstrated large variations in the genome of HeLa cells from different sources, thereby reducing their utility in cell and molecular biology.
DNA sequencing gives insight into the regulatory elements within the genome of every cell, and the variations in their activity in different cell types and individuals. **For instance, a particular gene may be permanently turned off in some tissues, while being constitutively expressed in others.** Similarly, those with susceptibility for a specific ailment may regulate a gene differently from those who are immune. These differences in the regulatory regions of

DNA can be demonstrated through sequencing and can give insight into the basis for a phenotype.
Recent advances have even allowed individual laboratories to study structural variations in the human genome – an undertaking that needed global collaboration two decades ago.

c. Forensics :

The ability to use low concentrations of DNA to obtain reliable sequencing reads has been extremely useful to the forensic scientist. In particular, the potential to sequence every DNA within a sample is attractive, especially since a crime scene often contains genetic material from multiple people. **HTS is slowly being adopted in many forensics labs for human identification.** In addition, recent advances allow forensic scientists to sequence the exome of a person after death, especially to determine the cause of death. For instance, death due to poisoning will show changes to the exome in affected organs. On the other hand, DNA sequencing can also determine that the deceased had a preexisting genetic ailment or predisposition. The challenges in this field include the development of extremely reliable analysis software, especially since the results of HTS cannot be manually examined.

Probable Questions:

1. What is RFLP ? Describe the procedure.
2. What is RAPD ? Describe the procedure.
3. How RFLP can be used in genetic mapping?
4. How RAPD can be used in genetic mapping?
5. How distance between two RFLP loci can be determined?
6. What are the limitations of RAPD ?
7. Describe AFLP .
8. What is restriction mapping? Describe the procedure.
9. Describe how FISH is performed?
10. Describe radiation hybrid mapping procedure.
11. Differentiate between Hierarchical genome shotgun and Whole genome shotgun.
12. What is high throughput sequencing ?
13. What is pyrosequencing ? Describe the process.
14. How Sanger sequencing is performed ?
15. What is dideoxynucleotide? What is its utility in Sanger sequencing ?
16. Write down the uses of DNA sequencing ?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
9. Primrose SB and Twyman RM. (2006) Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
10. Sambrook J and Russell D. (2001) Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Pre

Unit-III

Functional genomics: Study of gene interaction by the yeast two hybrid system; study of developmental regulation by using DNA-chips

Objective: In this unit you will know about functional genomics. Gene interaction study by yeast two hybrid system and microarray technique using DNA chips.

Functional Genomics

Genomics involves the mapping of an entire genome and, eventually, the determination of a species' complete DNA sequence. The amount of information found within a species' genome is enormous. The goal of functional genomics is to elucidate the roles of genetic sequences-DNA, RNA, and amino acid sequences-in a given species. In most cases, functional genomics is aimed at understanding gene function. At the genomic level, researchers can study genes as groups. For example, the information gained from a genome-sequencing project can help researchers study entire metabolic pathways. This provides a description of the ways in which gene products interact to carry out cellular processes. In addition, a study of genetic sequences can help to identify regions that play particular functional roles. For example, an analysis of certain species of bacteria helped to identify DNA sequences that promote the uptake of DNA during bacterial transformation. Because most genes encode proteins, a goal of many molecular biologists is to understand the functional roles of all the proteins a species produces. The entire collection of proteins a given cell or organism can make is called its proteome, and the study of the function and interactions of these proteins is termed proteomics. An objective of researchers in the field of proteomics is to understand the interplay among many proteins as they function to create cells and, ultimately, the traits of a given species.

Methods to Study Protein- Protein Interactions:

The operation of the genome can be evaluated by the study of proteome. Thus, by studying the functions of proteins, it is possible to understand how the genome operates and how a dysfunctional genome activity can result in disease states such as cancer. Proteomics broadly involves the methodology for characterizing the protein content of the cell. This can be done by protein electrophoresis, mass spectrometry etc.

Identification of protein-protein interaction is a recent approach to study proteome. The protein interaction maps can be constructed to understand the relation between the proteome and cellular biochemistry. Phage display and yeast two-hybrid system are commonly used to study protein- protein interactions.

Phage Display:

Phage display is a novel technique to evaluate genome activity with particular reference to identify proteins that interact with one another. It basically involves insertion of a foreign DNA into phage genome, and its expression as fusion product with a phage coat protein (Fig. 5.14A).

This is followed by screening of test protein by phage display library (Fig. 5.14B). The technique is briefly described below.

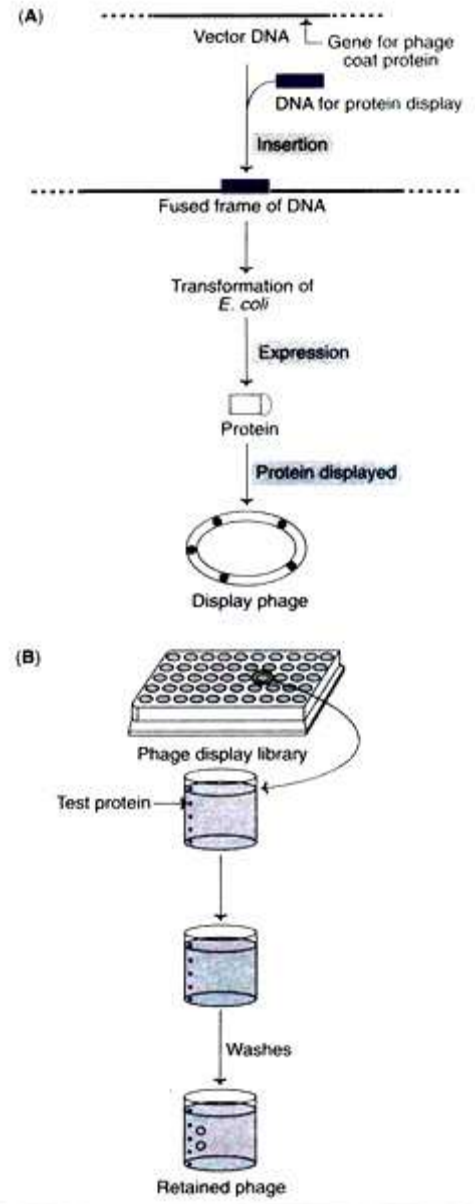


Fig. 5.14 : Elucidation of protein–protein interaction by phage display (A) Production of fusion protein displayed on phage (B) Screening of test protein by phage display library.

A special type of cloning vector such as a bacteriophage or filamentous bacteriophage (e.g. M13) are used for phage display. A fragment of DNA coding for the test protein is inserted into the vector DNA (adjacent to phage coat protein gene). After transformation of E. coli, this recombinant gene (fused frame of DNA) results in the synthesis of hybrid protein. The new protein is made up of the test protein fused with the phage coat protein. The phage particles produced in the transformed E. coli display the test protein in their coats.

The test protein interaction can be identified by using a phage display library. For this purpose, the test protein is immobilized within a well of a micro-titer tray, and the phage display library added. After several washes, the phages that are retained in the well are those displaying a protein that interacts with the test protein. Phage-displaying peptides can be isolated, based on their antibody-binding properties, by employing affinity chromatography. Several rounds of affinity chromatography and phage propagation can be used to enrich phages with desired proteins.

Phagemid display:

Phagemid in place of plasmid can also be used for the display of proteins. In fact, special types of phagemid display vectors have been developed for this purpose. Phage and phagemid display can be successfully used for selecting and engineering polypeptides with novel functions.

Yeast Two-Hybrid System:

When two proteins interact with each other, their corresponding genes are known as interacting genes. The yeast two-hybrid system uses a reporter gene to detect the physical interaction of a pair of proteins inside a yeast nucleus.

The two-hybrid method is based on the observation that most of the transcriptional proteins (i.e. the proteins involved in promoting transcription of a gene) contain two distinct domains—DNA binding domain and transcriptional activation domain. When these two domains are physically separated, the protein loses its activity. However, the same protein can be reactivated when the domains are brought together. These proteins can bind to DNA and activate transcription.

The target protein is fused to a DNA-binding domain to form a bait. When this target protein binds to another specifically designed protein namely the prey in the nucleus, they interact, which in turn switches on the expression of the reporter gene (Fig. 5.15). The reporter genes can be detected by growing the yeast on a selective medium.

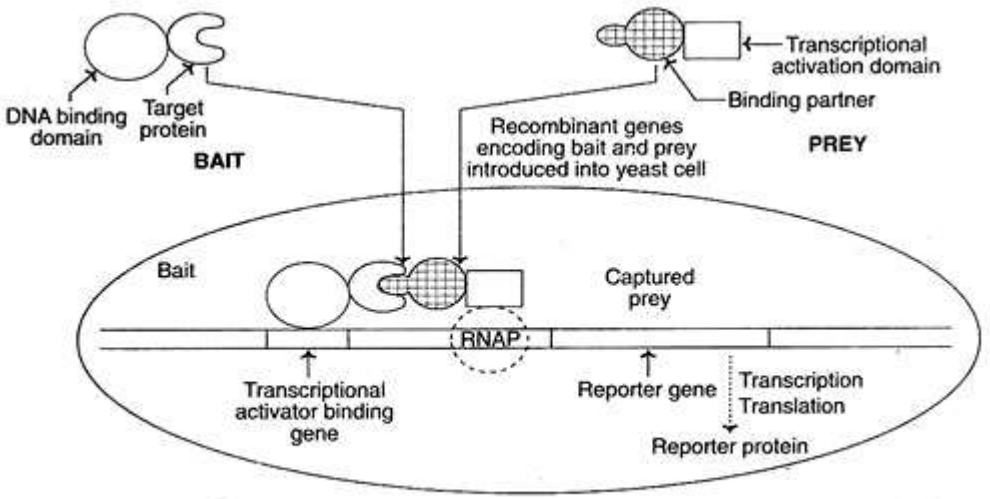


Fig. 5.15 : Elucidation of protein-protein interaction by yeast two-hybrid system (RNAP-RNA polymerase)

It is possible to generate the bait and prey fusion proteins by standard recombinant DNA techniques. A single bait protein is frequently used to fish out interacting partners among the collection of prey proteins. A large number of prey proteins can be produced by ligating DNA encoding the activation domain of a transcriptional activator to a mixture of DNA-fragments from a cDNA library.

The yeast two-hybrid system originally created by Fields and Song is a genetic system wherein the interaction between two proteins of interest is detected via the reconstitution of a transcription factor and the subsequent activation of reporter genes under the control of this transcription factor. In yeast, Galactose is imported into the cell and converted to galactose-6-phosphate by six enzymes (GAL1, GAL2, PGM2, GAL7, GAL10, MEL1) which are transcriptionally regulated by the proteins Gal80, Gal3, and Gal4, the latter of which plays the central role of DNA-binding transactivator. Gal80 binds Gal4 and inhibits its transcriptional ability. Gal3, in the presence of galactose, binds and causes a conformational change in Gal80, which then allows Gal4 to function as a transcriptional activator. Gal4, like other transcriptional activators, is a modular protein that requires both DNA-binding (BD) and activation domains (AD). The "two hybrid" technique exploits the fact that Gal4 cannot function as a transcriptional activator unless physically bound to an activation domain. Furthermore, it has been demonstrated that this interaction does not need to be covalent: an experiment was performed where the negative regulatory protein, Gal80, was fused with an activation domain to produce Gal80-AD, and was able to act as a transcriptional activator through its natural binding interaction with a Gal4 protein that was missing its own activation domain.

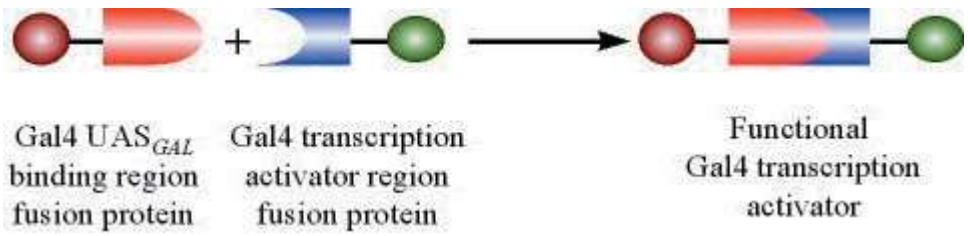


Fig 1: Gal 4 transcriptional activator.

In a classical assay system, a protein X is expressed as a fusion to a DNA binding domain (DBD). The DBD–X fusion is commonly termed the “bait.” Because of the affinity of the DBD for its operator sequences the bait is bound to a promoter element upstream of a reporter gene but does not activate it because it lacks an activation domain. A second protein Y is expressed as a fusion to an activation domain (AD) and is commonly termed the “prey.” The prey is capable of activating transcription but usually does not do so because it has no affinity for the promoter elements upstream of the reporter gene. If bait and prey are co-expressed and the two proteins X and Y interact, then a functional transcription factor is reconstituted at the promoter site upstream of the reporter gene. Consequently, transcription of the reporter gene is activated. Thus, in a yeast two-hybrid assay a protein-protein interaction is measured through the activation of one or several reporter genes in response to the assembly of a transcription factor by the said protein-protein interaction. In common yeast two-hybrid screening schemes the prey is usually replaced by a collection of unknown preys expressed from a cDNA or

genomic library. Screening of entire libraries against a defined bait may then lead to the discovery of novel interaction For large-scale screenings, two approaches are commonly used: the library screening approach, in which multiple baits are screened against a library, and the matrix approach, in which an array of defined preys is substituted for the library.

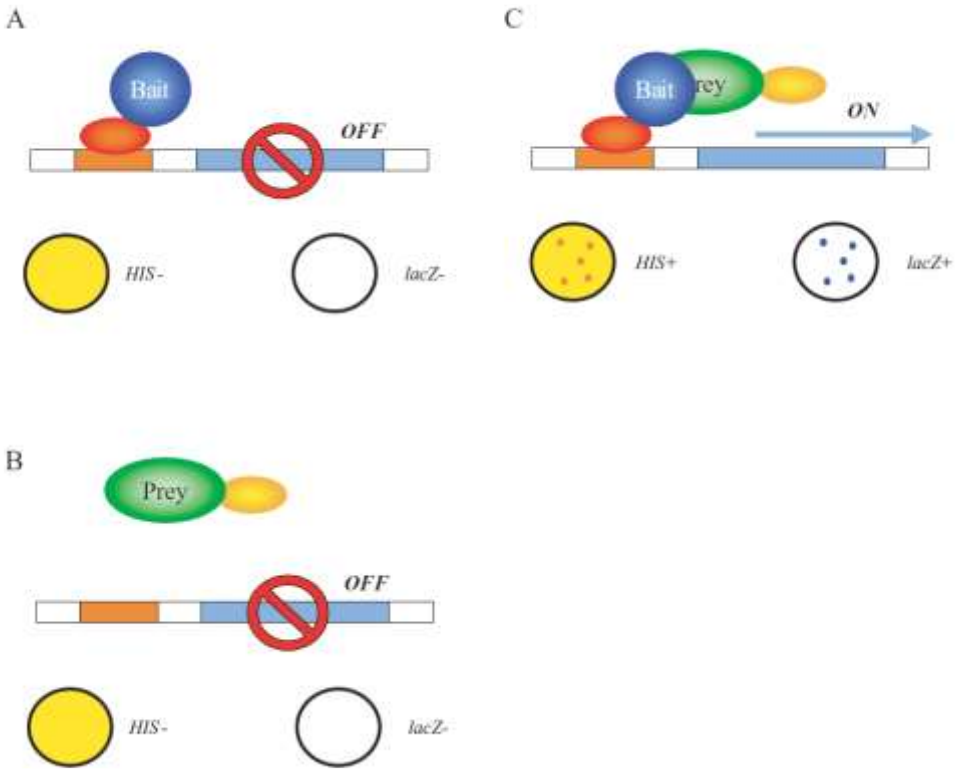


Fig 2: Yeast-Two-Hybrid system. The hybrid transcription factor is bound to the promoter upstream of the reporter gene and therefore activates transcription. The readout of the activated reporter gene is measured either as growth on selective medium (auxotrophic selection markers, such as *HIS3, URA3*, or *ADE2)* or in a colour reaction *(lacZ*). Yeast expressing only the DBD–bait or the AD–prey on its own do not grow on selective medium (*HIS*−) and do not display blue staining in a colour assay (*lacZ*-), whereas yeast harbouring an interacting DBD–bait and AD–prey display growth (*HIS*+) and blue colour (*lacZ*+).

Library Screening approach: In high throughput library approach a particular bait is expressed in a yeast reporter strain of the mating type a, whereas a collection of preys (the library) is transformed into a yeast reporter strain of the mating type *α*. The bait bearing strain is then mated with the mixture of library strains, and clones expressing an interaction pair are isolated on selective media. To determine the identity of the interacting prey, the library plasmid encoding it has to be isolated from the yeast strain and amplified in *Escherichia coli*. The region encoding the prey is then sequenced.

The Matrix screening Approach: In the matrix approach a collection of defined preys is used instead of a random collection of open reading frames (ORFs) or ORF fragments. Each prey is separately introduced into yeast and the transformants are arrayed on plates using a robot. A

bait-bearing strain of the opposite mating type is then mated with every prey-bearing strain and the resulting diploid strains are replicated onto selective medium. If a particular diploid within the array grows on selection medium, its prey must interact with the bait under investigation. As opposed to the library screen, no plasmid isolation or sequencing is necessary since the position of the growing diploid on the array identifies the prey it expresses. In essence, a matrix screen consists of a series of defined interactions between a bait and a numbers of prey, rather than a screen of a bait against a collection of unknown preys.

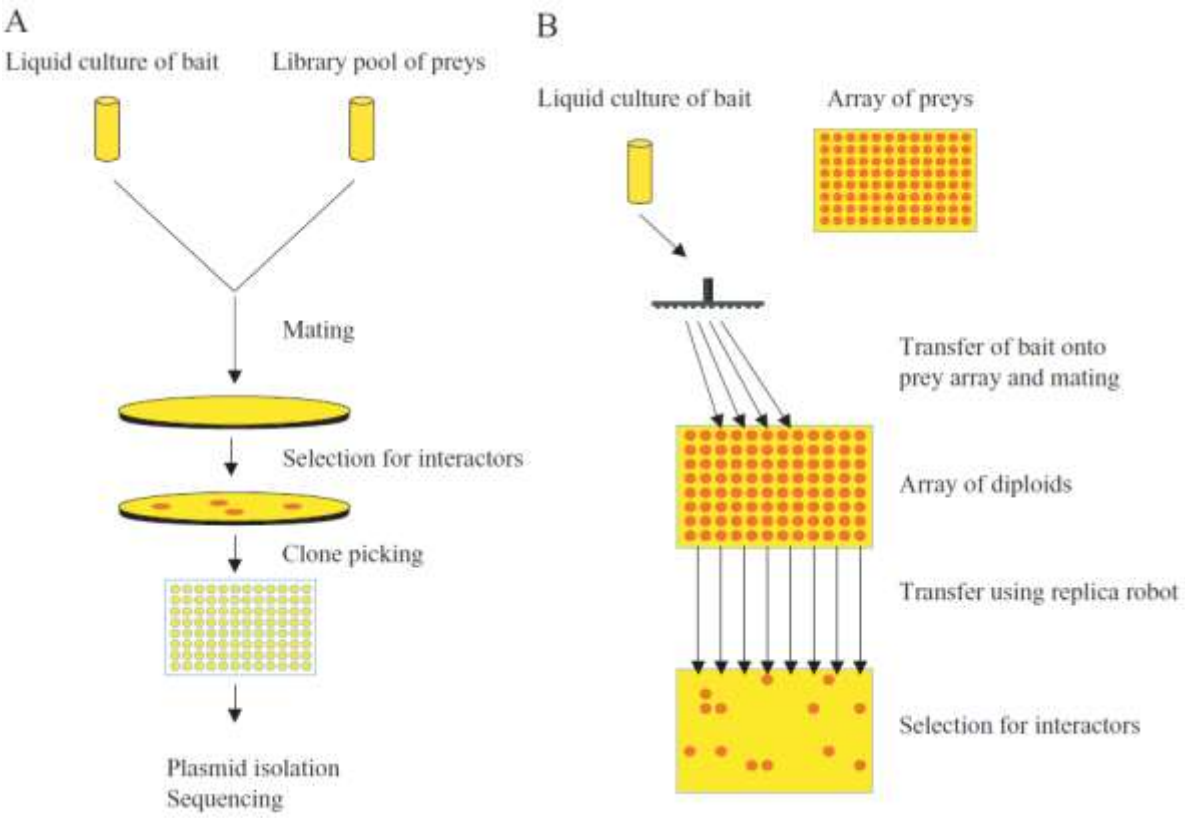


Fig 3: High throughput screening. (A) Library approach (B) Matrix approach

Yeast Three-Hybrid System:

The interactions between protein and RNA molecules can be investigated by using a technique known as yeast three-hybrid system.

DNA Microarray:

The DNA Microarray technology is used to determine the level of expression of many thousands of genes simultaneously. This new approach is used not for individual genetic loci, rather, for the analysis of genome-wide patterns of gene expression. Using DNA microarrays, it is possible to estimate the relative level of gene expression of each gene in the genome.

The DNA microarray or chip is a high density grid system, consisting of a flat solid substrate about the size of a postage stamp that can be used to detect hybridisation of target DNA under appropriate conditions. The chip contains 10,000 to 100,000 distinct spots, from 75 to 150 µm in diameter.

The spacing between spots on an array is usually 100 to 200 µm. Each spot contains a different immobilised DNA sequence that can be hybridised with DNA (or RNA) from a large number of different cells. Two types of chips are currently available: one, in which oligonucleotides have been synthesised directly on the chip, one nucleotide at a time, by automated procedures. These chips have hundreds of thousands of spots per array; second, chips in which double-stranded DNA sequences of 500 to 5000 base pairs have been deposited through drops by capillary action from miniaturized devices mounted on the movable head of a robotic workstation. These chips have tens of thousands of spots per array. The surface onto which DNA is spotted is critically important. The ideal surface immobilizes the target DNAs, and is compatible with stringent probe hybridisation conditions.

The procedure shown (Fig. 24.1) depicts only 6 spots in a chip, each of which contains a DNA sequence that serves as a probe for a different gene. Experimental cells are used for the extraction of cellular mRNA, and a control sample of mRNA from another source. The samples are subjected to reverse transcription to obtain DNA strands. In the experimental material, the primer for reverse transcription is tagged with a green fluorescent label, while primers of the control material receive red fluorescent label. After the DNA strands have been obtained in sufficient quantity, the fluorescent samples are mixed and hybridised with the DNA in the spots in the chip. The hybridisation is competitive because the two samples were mixed.

Therefore, the density of red and green strands bound to the chip is proportional to the concentration of red or green molecules in the mixture. Genes that are over-expressed in the experimental sample relative to the control will have more green strands hybridised to the spot, whereas those that are under-expressed in the experimental sample relative to the control will have more of red strands hybridised to the spot.

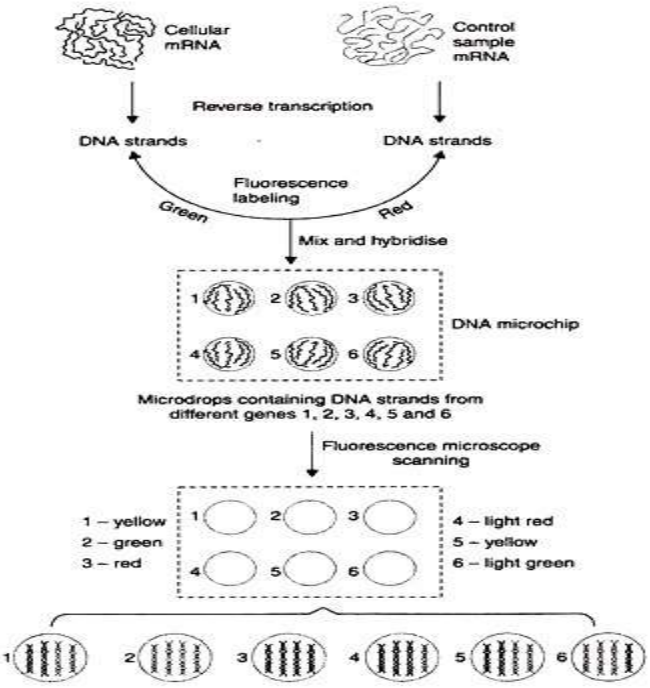


Fig. 24.1 Procedure for DNA microarrays. Six dried microdrops are introduced into a DNA microchip. Each drop contains immobilised DNA strands from a different gene numbered 1 to 6. These are hybridised with fluorescence-labelled DNA samples obtained by reverse transcription of cellular mRNA (green) and red labelled control DNA sample. Competitive hybridisation of green (experimental) and red (control) label is proportional to the *relative* abundance of each mRNA in the sample. The intensity of red and green fluorescence is analysed by microscopy and interpreted as overexpression, underexpression and equal expression of the gene depending on the intensity of red, green, orange, yellow-green and yellow fluorescence.

The intensity of fluorescence is viewed by placing the chip under a laser scanning microscope or a fluorescence microscope that scans each pixel, which is the smallest discrete unit in a visual image. The intensity of fluorescent label is recorded. The signals are synthesised to produce a signal value for each spot in the microarray.
The signals indicate the relative levels of gene expression through colour. Green or yellow green indicate over-expression in experimental sample, while red or orange indicates under-expression in experimental sample. Yellow indicates equal expression in both experimental and control samples.
DNA microarray technology is useful for study of large number of cells growing under different conditions, at different developmental stages, or at different stages of a disease. Besides detection of gene expression, this technology can be used to detect mutations and polymorphisms, to map genomic DNA clones, and to compare the gene expression pattern in normal and diseased tissues.

Fabrication:
Microarrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto glass slides, photolithography using pre-made masks, photolithography using dynamic micro-mirror devices, ink-jet printing, or electrochemistry on microelectrode arrays.
DNA microarrays can be used to detect RNAs that may or may not be translated into active proteins. Scientists refer to this kind of analysis as "expression analysis" or expression profiling. Since there can be tens of thousands of distinct probes on an array, each microarray experiment can accomplish the equivalent number of genetic tests in parallel. Arrays have, therefore, dramatically accelerated many types of investigations. The use of microarrays for gene expression profiling was first published in 1995 (Science) and the first complete eukaryotic genome {Saccharomyces cerevisiae) on a microarray was published in 1997 (Science).

1. Spotted Microarrays*:*
In spotted microarrays (or two-channel or two-colour microarrays), the probes are oligonucleotides, cDNA or small fragments of PCR products that correspond to mRNAs and are spotted onto the microarray surface. This type of array is typically hybridized with cDNA from two samples to be compared (e.g., diseased tissue versus healthy tissue) that are labelled with two different fluorophores (e.g., Rhodamine (Cyanine 5, red) and Fluorescein (Cyanine 3, green)). The two samples are mixed and hybridized to a single microarray that is then scanned in a microarray scanner to visualize fluorescence of the two fluorophores. Relative intensities of each fluorophore are then used to identify up-regulated and down-regulated genes in ratio-based analysis. Absolute levels of gene expression cannot be determined in the two-colour array, but relative differences in expression among different spots (= genes) can be estimated with some oligonucleotide arrays.

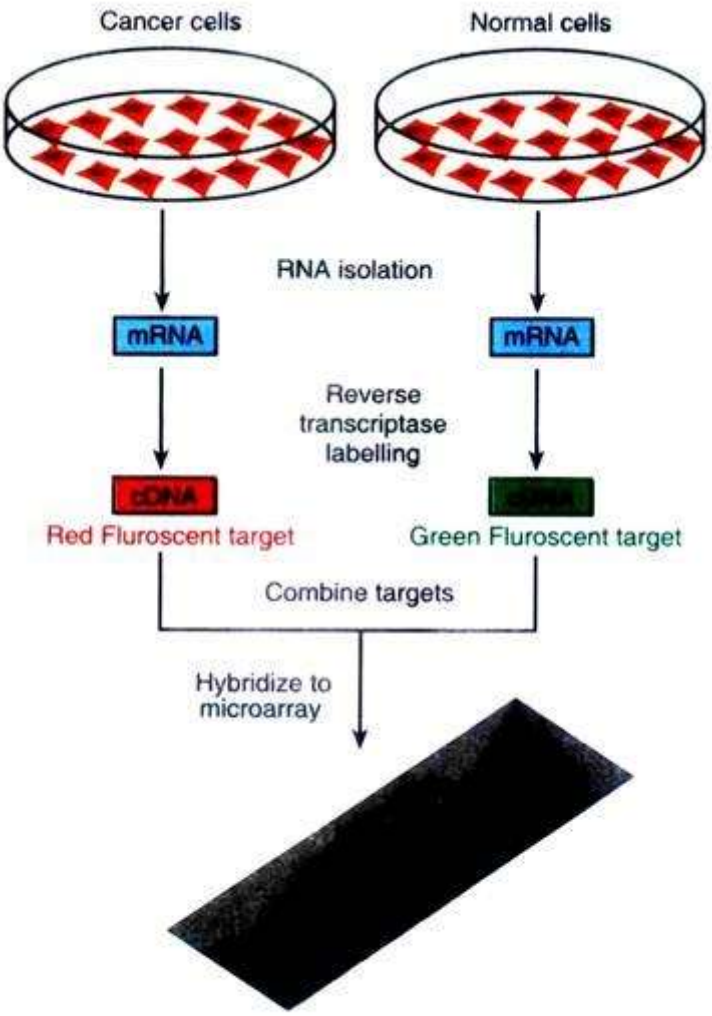


Fig. 16.2: Diagram of typical dual-colour microarray experiment

2. Oligonucleotide Microarrays:

In oligonucleotide microarrays (or single-channel microarrays), the probes are designed to match parts of the sequence of known or predicted mRNAs. There are commercially available designs that cover complete genomes from companies such as GE Healthcare, Affymetrix, Ocimum Bio-solutions, or Agilent. These microarrays give estimations of the absolute value of gene expression and, therefore, the comparison of two conditions requires the use of two separate micro- arrays.

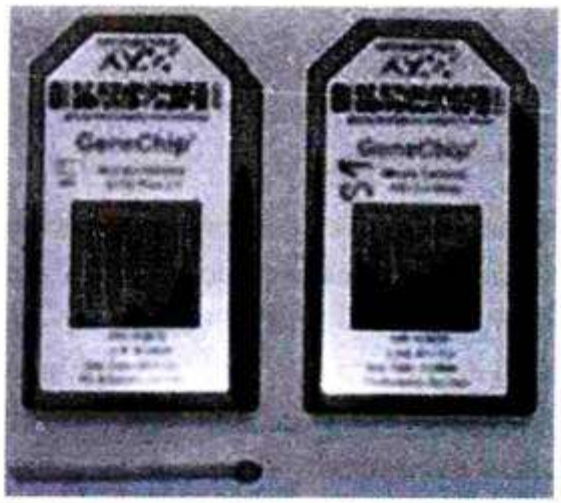


Fig. 16.3: Two Affymetrix chips

Oligonucleotide Arrays can be either produced by piezoelectric deposition with full length oligonucleotides or in situ synthesis. Long Oligonucleotide Arrays are composed of 60-mers, or 50-mers and are produced by ink-jet printing on a silica substrate. Short Oligonucleotide Arrays are composed of 25-mer or 30-mer and are produced by photolithographic synthesis (Affymetrix) on a silica substrate or piezoelectric deposition (GE Healthcare) on an acrylamide matrix. More recently, Maskless Array Synthesis from NimbleGen Systems has combined flexibility with large numbers of probes. Arrays can contain up to 390,000 spots, from a custom array design. New array formats are being developed to study specific pathways or disease states for a systems biology approach.

Oligonucleotide microarrays often contain control probes designed to hybridize with RNA spike-ins. The degree of hybridization between the spike-ins and the control probes is used to normalize the hybridization measurements for the target probes.

Genotyping Microarrays:

DNA microarrays can also be used to read the sequence of a genome in particular positions. SNP microarrays are a particular type of DNA microarrays that are used to identify genetic variation in individuals and across populations.

Short oligonucleotide arrays can be used to identify the single nucleotide polymorphisms (SNPs) that are thought to be responsible for genetic variation and the source of susceptibility to genetically caused diseases. Generally termed genotyping applications, DNA microarrays may be used in this fashion for forensic applications, rapidly discovering or measuring genetic predisposition to disease, or identifying DNA-based drug candidates. These SNP microarrays are also being used to profile somatic mutations in cancer, specifically loss of heterozygosity events and amplifications and deletions of regions of DNA. Amplifications and deletions can also be detected using comparative genomic hybridization, or aCGH, in conjunction with microarrays, but may be limited in detecting novel Copy Number Polymorphisms, or CNPs, by probe coverage.

Re-sequencing arrays have also been developed to sequence portions of the genome in individuals. These arrays may be used to evaluate germ line mutations in individuals, or somatic mutations in cancers. Genome tiling arrays include overlapping oligonucleotides designed to blanket an entire genomic region of interest. Many companies have successfully designed tiling arrays that cover whole human chromosomes.

Microarrays and Bioinformatics:

1. Experimental Design:

Due to the biological complexity of gene expression, the considerations of experimental design that are discussed in the expression profiling article are of critical importance if statistically and biologically valid conclusions are to be drawn from the data.

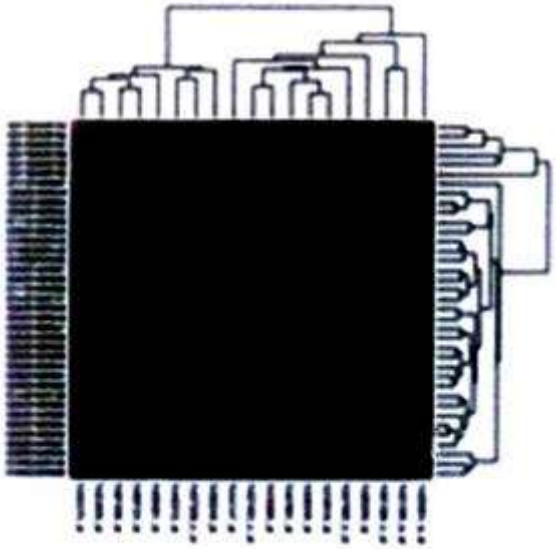


Fig. 16.4: Gene expression values from microarray experiments can be represented as heat maps to visualize the result of data analysis

There are three main elements to consider when designing a microarray experiment.

First, replication of the biological samples is essential for drawing conclusions from the experiment.

Second, technical replicates (two RNA samples obtained from each experimental unit) help to ensure precision and allow for testing differences within treatment groups. The technical replicates may be two independent RNA extractions or two aliquots of the same extraction.

Third, spots of each cDNA clone or oligonucleotide are present at least as duplicates on the microarray slide, to provide a measure of technical precision in each hybridization. It is critical that information about the sample preparation and handling is discussed in order to help identify the independent units in the experiment as well as to avoid inflated estimates of significance.

2. Standardization:

The lack of standardization in arrays presents an interoperability problem in bioinformatics, which hinders the exchange of array data. Various grass-roots open-source projects are attempting to facilitate the exchange and analysis of data produced with non-proprietary chips.

a. The "Minimum Information about a Microarray Experiment" (MIAME) checklist helps define the level of detail that should exist and is being adopted by many journals as a requirement for the submission of papers incorporating microarray results. MIAME describes the minimum required information for complying experiments, but not its format. Thus, as of 2007, whilst many formats can support the MIAME requirements there is no format which permits verification of complete semantic compliance.

b. The "MicroArray Quality Control (MAQC) Project" is being conducted by the FDA to develop standards and quality control metrics which will eventually allow the use of MicroArray data in drug discovery, clinical practice and regulatory decision-making.

3. The MicroArray and Gene Expression (MAGE) group is working on the standardization of the representation of gene expression data and relevant annotations.

3. Statistical Analysis:

The analysis of DNA microarrays poses a large number of statistical problems, including the normalization of the data. There are dozens of proposed normalization methods in the published literature; as in many other cases where authorities disagree, a sound conservative approach is to try a number of popular normalization methods and compare the conclusions reached; how sensitive are the main conclusions to the method chosen?

From a hypothesis-testing perspective, the large number of genes present on a single array means that the experimenter must take into account a multiple testing problem; even if the statistical P-value assigned to a given gene indicates that it is extremely unlikely that differential expression of this gene was due to random rather than treatment effects, the very high number of genes on an array makes it likely that differential expression of some genes represents false positives or false negatives.

Statistical methods tailored to microarray analyses have recently become available that assess statistical power based on the variation present in the data and the number of experimental replicates, and can help minimize type I and type II errors in the analyses.

A basic difference between microarray data analysis and much traditional biomedical research is the dimensionality of the data. A large clinical study might collect 100 data items per patient for thousands of patients. A medium-size microarray study will obtain many thousands of numbers per sample for perhaps a hundred samples. Many analysis techniques treat each sample as a single point in a space with thousands of dimensions, then attempt by various techniques to reduce the dimensionality of the data to something humans can visualize.

4. Relation between Probe and Gene:

The relation between a probe and the mRNA that it is expected to detect is problematic. On the one hand, some mRNAs may cross-hybridize probes in the array that are supposed to detect another mRNA. On the other hand, probes that are designed to detect the mRNA of a particular gene may be relying on genomic EST information that is incorrectly associated with that gene.

Public Databases of Microarray Data:

Database	Microarray Experiment Sets	Sample Profiles	As of Date
Gene Expression Omnibus - NCBI	5366	134669	April 1, 2007
Stanford Microarray database	12742	?	April 1, 2007
UNC Microarray database	~31	2093	April 1, 2007
MUSC database	~45	555	April 1, 2007
ArrayExpress at EBI	1643	136	April 1, 2007
caArray at NCI	41	1741	November 15, 2006

Online Microarray Data Analysis Programs and Tools:

Several Open Directory Project categories list online microarray data analysis programs and tools:

i. Bioinformatics: Online Services:
Gene Expression and Regulation at the Open Directory Project

ii. Gene Expression:
Databases at the Open Directory Project

iii. Gene Expression:
Software at the Open Directory Project

iv. Data Mining:
Tool Vendors at the Open Directory Project

v. Bio-conductor:
Open source and open development software project for the analysis and comprehension of genomic data

vi. Genevestigator:
Web-based database and analysis tool to study gene expression across large sets of tissues, developmental stages, drugs, stimuli, and genetic modifications.

DNA Chip and Development study:
The mechanistic basis of metazoan development represents one of the unsolved mysteries of biology: how does a single fertilized egg, through successive cell divisions and differentiation events, mature into an adult organism? The fruitfly *Drosophila melanogaster* has been a pioneering model organism for geneticists and developmental biologists for many decades. Drosophilogists have been quick to exploit the power of genome-wide expression profiling using DNA microarrays. One notable example is the study of the expression of 4028 genes analysed in wild-type flies throughout *Drosophila* development during 66 sequential time periods. These included sampling RNA at fertilization, embryonic, larval and pupal periods as well as the first 30 days of adulthood. Each experimental sample was compared with a common

reference sample, allowing the relative abundance of any transcript to be determined at every developmental stage. The analysis of such a huge amount of data conventionally proceeds by the use of algorithms that group or cluster genes according to similarity in their expression profiles.

The analysis of the *Drosophila* dataset revealed that, despite the use of whole animals, it was possible to discern expression profiles in specific organs, as well as those associated with particular biological processes. For example, one cluster of 23 genes included eight known to be expressed in terminally differentiated muscle. The profile of this cluster has two peaks of expression, one coinciding with the larval stage and a second with adult muscle development. Initiation of larval muscle development is regulated by the basic helix–loop–helix (bHLH) transcription factor Twist, which induces expression of *dMef 2*, which itself encodes a MADS box transcription factor regulating the transcription of muscle differentiation genes. Crucially, this muscle-specific regulatory hierarchy was recapitulated in the microarray data: the peak of *twist* expression preceded that of *dMef 2*, which preceded transcription of genes in a muscle differentiation cluster. Moreover, 15 of the 23 genes in this latter cluster contained pairs of predicted dMEF2-binding sites. Similar clusters were identified revealing coordinate expression profiles associated with particular biochemical and cellular functions, including mitochondrial proteins, components of the 26S proteasome complex and cytoskeletal/neuronal factors.

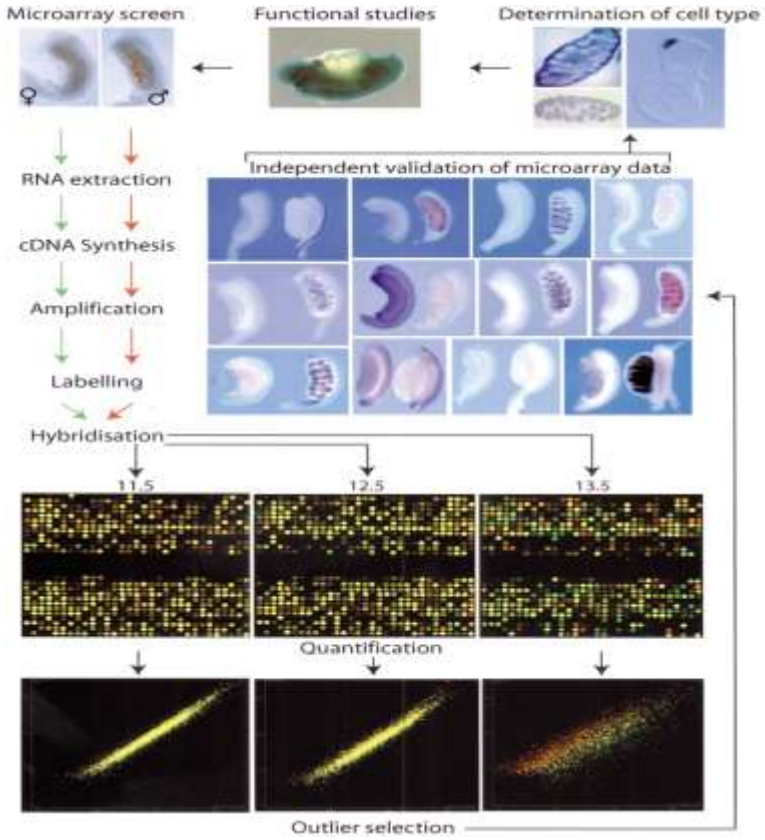


Fig 5: A screen for genes expressed in a sexually dimorphic fashion during mouse gonad and mesonephros development using DNA microarrays.

Global transcriptional information during morphogenesis was also readily available: the vast majority of genes (>88%) that exhibit transcriptional modulation during the stages analysed are expressed during the first 20 h of development, before the end of embryogenesis. A total of 2103 changed during embryogenesis, 445 changed during larval life, 646 during the pupal stage and 118 during adult life. The transcript levels of only 16 genes changed significantly during the adult time period sampled. These data suggest a strong association between modulation of transcriptional activity and morphogenesis.

The pioneering experiments in invertebrates suggest that the notion that gene expression profiles alone do not reveal biological function needs to be re-examined. Surveying gene expression under a wide range of conditions and tissues, in wild-type and mutant animals, seems to transform the significance of data that on a smaller scale would be considered descriptive. Of course, for any individual gene residing within a 'functionally loaded' cluster the task remains to determine the phenotypic consequences of its mutation. Yet perhaps such experiments should be seen as complementing our understanding of that gene's function developed by other means, rather than being exclusively definitive thereof: particularly given the high frequency with which no clear phenotype is observed after mutagenesis. To infer function from gene expression profiles is not mere speculation if the design of the experiment and the complexity of the dataset permit otherwise

Studies were done in the mammalian embryo at a genome-wide level throughout its development, in a manner reminiscent of those discussed in invertebrates. Given the widespread accessibility of microarray technology today, the observations and analysis are very complex, involving references to both technical and 'cultural' issues. The most common technical problem concerns the small amounts of RNA available from standard dissections of mammalian embryos. By 'cultural', we mean the familiarity that developmental biologists have with in situ hybridization (ISH), their relative lack of familiarity with microarrays and the common attitude that descriptions of gene expression patterns support only speculation about function. However, these remarks are equally applicable to developmental biologists using flies and worms as a model. The use of DNA microarrays to examine mammalian development is a small but rapidly growing field of study. It is currently dominated by the exploitation of arrays to perform screens for molecules involved in particular developmental processes.

Systematic genome-wide studies of mammalian development using microarrays stand out due to their rarity. Studies in mouse and the analysis of the expression of 18 816 mouse genes in 49 different embryonic and adult tissues, permitted some clustering of genes pertinent to the development of specific tissues, such as the central nervous system. However, the limited number of embryonic samples, totalling 11, means that this study falls short of providing a transcriptional profile of mouse development. Perhaps due to the relative complexity of the mammalian embryo, more familiar are studies aimed at profiling expression at specific embryonic stages or in specific embryonic tissues, including (without attempting to be comprehensive): 12.5 days post coitum (dpc) mouse placenta, mouse retina, mouse lung, mouse mammary gland, preimplantation mouse embryos, mouse hippocampus, and mouse B cells. Developmental biologists have also been quick to adapt familiar techniques for the purposes of exploiting microarray technology, including the use of cell line models and organ cultures too.

Applications of these Arrays include:

1. mRNA or gene expression profiling:

Monitoring expression levels for thousands of genes simultaneously is relevant to many areas of biology and medicine, such as studying treatments, disease, and developmental stages. For example, microarrays can be used to identify disease genes by comparing gene expression in diseased and normal cells.

2. Comparative genomic hybridization (Array CGH):

Assessing large genomic rearrangements.

3. SNP detection arrays:

Looking for single nucleotide polymorphism in the genome of populations.

4. Chromatin immunoprecipitation (ChIP) studies:

Determining protein binding site occupancy throughout the genome, employing ChlP-on-chip technology.

Probable Questions:

1.How phage display technique can be used for protein protein interaction assay?

2. What is yeast two hybrid system? How it is used to determine protein protein interaction?

3. What is DNA chip?

4. State the basic principle of DNA microarray analysis.

5. What is spotted microarray? Explain.

6. What is oligonucleotide microarray? Explain .

7. How DNA chip can be used in developmental study?

8. What are the applications of DNA microarray?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
9. Primrose SB and Twyman RM. (2006) Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.

Unit-IV

Comparative genomics: Orthologuos, Paraloguos and Gene displacement, Phylogenetic finger printing

Orthologous and paralogous genes are two types of homologous genes, that is, genes that arise from a common DNA ancestral sequence. Orthologous genes diverged after a speciation event, while paralogous genes diverge from one another within a species. Put another way, the terms orthologous and paralogous describe the relationships between genetic sequence divergence and gene products associated with speciation or genetic duplication.

Understanding Homologous Genes

Orthologous and paralogous genes are different types of homologous genes. Homologous genes are two or more genes that descend from a common ancestral DNA sequence. An example of homologous genes are the genetic codes underlying a bat wing and a bear arm. Both retain similar features and are utilized in similar manners. These traits, which were passed down from their last common ancestor, have adaptive pressures that may lead to variations within the gene. The point or event in evolutionary history that accounts for the DNA sequence variation within the gene determines whether the homologous genes are considered ‘ortho’ or ‘para’.

Orthologous Genes

Orthologous genes are homologous genes that diverged after evolution gives rise to different species, an event known as speciation. The genes generally maintain a similar function to that of the ancestral gene that they evolved from. In this type of homologous gene, the ancestral gene and its function is maintained through a speciation event, though variations may arise within the gene after the point in which the species diverged.

Paralogous Genes

Paralogous genes are homologous genes that have diverged within one species. Unlike orthologous genes, a paralogous gene is a new gene that holds a new function. These genes arise during gene duplication where one copy of the gene receives a mutation that gives rise to a new gene with a new function, though the function is often related to the role of the ancestral gene.

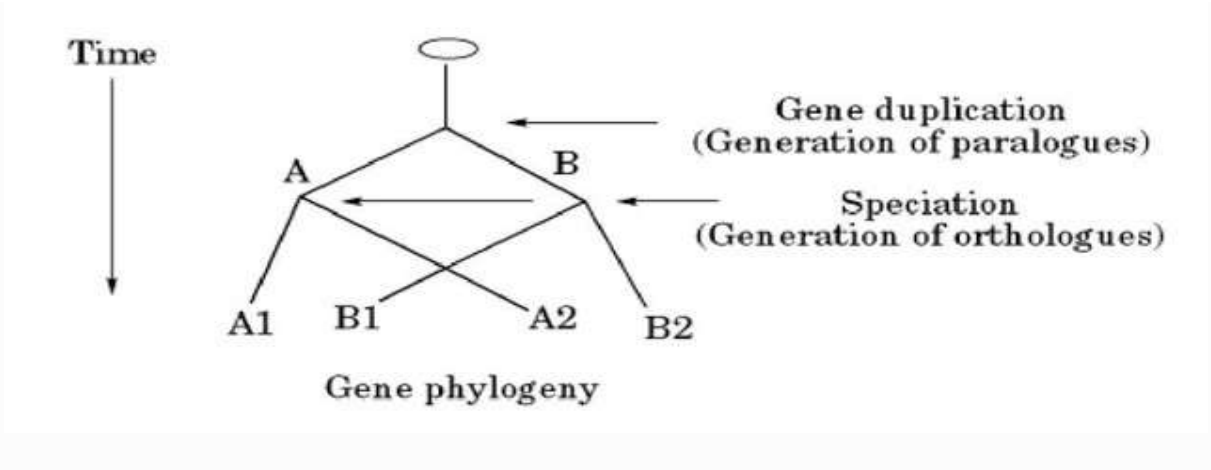


Figure 1. Generation of orthologous and paralogous genes

Examples of Paralogous and Orthlogous Genes

The genes that produce the haemoglobin and myoglobin proteins are homologous genes that have both orthologous and paralogous relationships. Both humans and dogs hold the genes for both haemoglobin and myoglobin proteins, indicating that the haemoglobin and myoglobin genes evolved before human's and dog's last common ancestor. Myoglobin arose in this ancestral species as a paralogous gene to haemoglobin; a mutation in the haemoglobin gene during a duplication event resulted in a separate myoglobin gene that carries out a new, yet similar, function. Since divergence in human and dog haemoglobin did not occur until after speciation, these genes are orthologous. Human myoglobin and dog haemoglobin, however, are homologous genes that are neither paralogous or orthologous.

Gene displacement

Comparative genomics has revealed many examples in which the same function is performed by unrelated or distantly related proteins in different cellular lineages. In some cases, this has been explained by the replacement of the original gene by a paralogue or non-homologue, a phenomenon known as non-orthologous gene displacement. Such gene displacement probably occurred early on in the history of proteins involved in DNA replication, repair, recombination and transcription (DNA informational proteins), i.e. just after the divergence of archaea, bacteria and eukarya from the last universal cellular ancestor (LUCA). This would explain why many DNA informational proteins are not orthologues between the three domains of life. However, in many cases, the origin of the displacing genes is obscure, as they do not even have detectable homologues in another domain. I suggest here that the original cellular DNA informational proteins have often been replaced by proteins of viral or plasmid origin. As viral and plasmid-encoded proteins are usually very divergent from their cellular counterparts, this would explain the puzzling phylogenies and distribution of many DNA informational proteins between the three domains of life.

Non-orthologous gene displacement in the evolution of DNA informational proteins Most DNA replication proteins that are functionally analogous in bacteria and eukarya have either no sequence similarities between these two domains or only limited similarities restricted to a

few amino acids involved in active sites. For example, the polymerization subunit of the bacterial replicase, DNA polymerase III, has no sequence similarity at all to its eukaryotic counterparts, the catalytic subunits of DNA polymerases a, d and e. In fact, bacterial and eukaryotic replicases belong to two distinct DNA polymerase families, C and B, respectively (Ito and Braithwaite, 1991). Similarly, although the bacterial and eukaryotic initiator proteins (DnaA and ORC/CDC6 respectively) belong to the same superfamily (the AAA‡ superfamily described recently by Neuwald et al., 1999), they are very distantly related, only sharing a common ATP binding site. The situation is similar for primases, helicases and ligases. Most bacterial replicative proteins thus have no clear-cut orthologues in eukarya and vice versa (Mushegian and Koonin, 1996; Koonin and Galperin, 1997; Galperin et al., 1998). To a lesser extent, a similar situation can be observed in repair/recombination and transcription systems, as some bacterial proteins involved in these processes have no homologues in eukarya, and vice versa (Aravind et al., 1999). In contrast, most functionally analogous proteins involved in translation (e.g. ribosomal proteins, elongation factors, tRNA synthetases) are clearly orthologues in bacteria and eukarya. From comparative genomics (Brown and Doolittle, 1997; Olsen and Woese, 1997; Aravind et al., 1999), it turns out that all bacterial replicative proteins and many bacterial proteins involved in repair, recombination and transcription also have no detectable homologues in archaea. In contrast, some eukaryotic proteins involved in these mechanisms have readily detectable archaeal homologues, which are most probably their orthologues. To explain why DNA replicative proteins were so different in bacteria on one side and in eukarya/archaea on the other, Koonin and colleagues suggested that LUCA was a member of the RNA world and that DNA informational proteins originated independently in the bacterial and eukaryal (archaeal) lineages from unrelated RNA informational proteins (Mushegian and Koonin, 1996; Aravind et al., 1999). However, not all DNA informational proteins are unrelated between bacteria and the two other domains. Some of them are indeed clearly homologues in all extant cellular organisms (e.g. large subunits of DNA-dependent RNA polymerases, ribonucleotide reductases, Topo IA, recombinases of the RecA family and a few other DNA recombination/repair proteins), suggesting to several authors that these proteins dealing with DNA were already present in a LUCA with a DNA genome (Lazcano et al., 1992; Forterre et al., 1993; 1994; Edgell and Doolittle, 1997; Riera et al., 1997). If one realizes that DNA is a speci®c form of modi- ®ed RNA (thymine-dRNA), I think that it is indeed dif®cult to imagine that DNA and DNA-processing enzymes have been independently invented twice in two lineages, as many other types of nucleic acids could have been produced from RNA modification. An alternative to the RNA-LUCA hypothesis would be that DNA informational proteins have diverged to such an extent from their ancestors in LUCA that their orthology can no longer be recognized between bacteria and eukarya/archaea. However, it is not clear why DNA informational proteins should have evolved much more rapidly than proteins involved in the translation machinery. Moreover, one should explain why, despite their involvement in common mechanisms, some of them have evolved very rapidly to become apparently unrelated between bacteria and the other two domains, whereas others have evolved slowly, with their orthology still recognizable in all three domains. It has been argued that the replicative mechanism was very primitive in LUCA and was only re®ned after the divergence of the three domains (Olsen and Woese, 1997). This could indeed have produced a core of proteins present in the primitive system and still homologues in the three domains and a set of

`re®nement' proteins, added later independently in each lineage and, thus, unrelated from one domain to another. However, a primitive replicative system should contain at least a replicase, a primase and a helicase, three proteins that are clearly non-orthologues between bacteria and eukarya/archaea, whereas putative `refinement' proteins, such as clamp loading factors, are homologues and possibly orthologues in the three domains! It has been suggested recently that informational proteins were so different between archaea and bacteria because archaeal proteins evolved to escape antibiotics produced by Gram-positive relatives of archaeal ancestors (Gupta, 1998). But this does not explain why these differences are especially high in the case of replicative proteins, as proteins involved in translation are, in fact, more often the targets of antibiotics. Furthermore, resistance to antibiotics usually involves only minor modifications that are restricted to a limited part of the protein. For example, archaeal DNA polymerases of the B family can be either sensitive or resistant to the drug aphidicolin in the same genus, despite a high degree of sequence identity (Forterre et al., 1994). Finally, although most archaeal and eukaryal replicative proteins are orthologues, a few archaeal replicative proteins are also unrelated or very distantly related to their eukaryal functional analogues, and vice versa. For example, the archaeal Topo VI, which is probably involved in chromosome segregation, and its functional counterparts in eukaryotes, Topo II, belong to different DNA topoisomerase II families (Bergerat et al., 1997). Similarly, eukaryal Topo IB, which is involved in the relaxation of positive superturn at the eukaryotic replication fork, has no homologues in archaeal genomes. Such an erratic pattern of relationships between archaeal and eukaryal replicative proteins cannot be explained by the LUCA-RNA theory because, as many archaeal and eukaryal replicative proteins are most probably orthologues, the common ancestor of archaea and eukarya was certainly a DNA-based organism. It cannot be explained either by differences in evolutionary rates, as, for example, eukaryotic Topo IB (a type I DNA topoisomerase) is not even structurally and mechanistically related to its bacterial and archaeal proteins that perform the same function in DNA replication (which are both type II DNA topoisomerases) (Forterre et al., 1994). In that case, it is clear that the puzzling distribution of DNA topoisomerase between archaea and eukarya can only be explained by non-orthologous gene displacement.

The term non-orthologous gene displacement has been coined recently by Koonin to describe the presence of nonorthologous proteins (unrelated or paralogues) for the same function in different organisms (Mushegian and Koonin, 1996; Koonin and Galperin, 1997; Koonin et al., 1996; 1997) (Fig. 1A). Now that several completely sequenced archaeal and bacterial genomes are available, it is clear that the displacement of proteins responsible for essential functions by evolutionary unrelated or distantly related proteins has been extensive in the archaeal and eukaryal domains, and even more so between domains (Koonin et al., 1997; Doolittle, 1998a). A priori, one would have thought that non-orthologous gene displacement should be limited to proteins that do not physically interact with other proteins, because it is difficult to envisage the replacement of a protein that physically interacts with several partners by a phylogenetically distantly related or unrelated protein (Jain et al., 1999). However, although DNA informational proteins are often part of macromolecular complexes, many well-documented cases of non-orthologous displacement between various lineages of a single domain have now been reported for genes encoding such proteins. For example, most of the primosome components are non-homologous between E. coli and B. subtilis, whereas one of them (PriA) and other parts of the

replication apparatus (DNA helicase, primase, replicase) are clearly orthologues (Bruand et al., 1995; Kunst et al., 1997). E. coli and B. subtilis also use analogous but non-homologous systems to produce single-stranded DNA for genetic recombination, the RecBCD and the AddAB/RexAB helicase/ exonuclease respectively (El Karoui et al., 1998), whereas they both use orthologous RecA to complete the recombination pathway. This example is highly significant as some Gram-positive bacteria possess the RecBCD system, whereas others have the AddAB/RexAB system, indicating that non-orthologous displacement of DNA informational proteins even occurred during the diversification of Gram-positive bacteria. The absence of homologues of the eukaryotic DNA replication initiator proteins ORC1/ CDC6 in the genome of the archaeon *Methanococcus jannaschii* also suggests recent non-orthologous displacement. Indeed, this protein, which is present in all other completely sequenced archaeal genomes, probably plays an essential role in the initiation of archaeal DNA replication (Lopez et al., 1999a). Accordingly, its function should have been taken over by another unknown protein in *M. jannaschii* (Bernander, 1998). In all these cases, one cannot explain the puzzling pattern of protein distribution observed inside one domain by divergent rates of evolution or by the RNA-LUCA hypothesis! If non-orthologous gene displacement of DNA informational proteins thus clearly occurred between both archaea and eukarya (as in the case of type II DNA topoisomerases) and after the diversification of each domain in multiple lineages it most likely that non-orthologous gene displacement also occurred between bacteria and archaea/eukarya in the replicative, repair, recombination and transcription apparatus, explaining why some DNA informational proteins are unrelated between bacteria and archaea/eukarya. This hypothesis is more parsimonious than either the RNA-LUCA hypothesis or the replicative protein fast evolution hypothesis, as it gives the same explanation (nonorthologous gene displacement) for the presence of phylogenetically unrelated functional analogues between domains and between different lineages of a domain, whereas competitive hypotheses involved two unrelated explanations. The idea that non-orthologous gene displacement in general has played a major role in the history of DNA informational proteins is supported by many examples of functional complementation that have been observed experimentally in studying DNA replication. For example, while the removal of RNA primers from Okazaki fragments is normally performed in E. coli by the 58 to 38 exonuclease activity of DNA polymerase I, it can be done by RNase H in mutants lacking this exonuclease activity (Ogawa and Okazaki, 1984). Similarly, E. coli DNA polymerase II can be used as replicase in some E. coli DNA polymerase III mutants (Rangarajan et al., 1997). The participation of DNA polymerase II at the E. coli replication fork is in line with the observation that, despite belonging to different DNA polymerase families (B and C respectively), both E. coli DNA polymerases II and III can interact in vitro with the bacterial processivity factor b-clamp and clamp-loading factors (Bonner et al., 1992). Finally, a well-known example of functional complementation is the displacement of a thermosensitive E. coli DnaA protein by the initiator protein of another replicon at non-permissive temperature (Tresguerres et al., 1975). In that case, the E. coli mutant is rescued using the replication origin of an integrated replicon as a new oriC.

DNA Fingerprinting:

DNA fingerprinting is based on sequence polymorphisms, slight sequence differences (usually single base-pair changes) between individuals, 1 bp in every 1,000 bp, on average. Each difference from the prototype human genome, sequence (the first one obtained) occurs in some fraction of the human population; every individual has some differences. Some of the sequence changes affect recognition sites for restriction enzymes, resulting in variation in the size of DNA fragments produced by digestion with a particular restriction enzyme.

These variations are restriction fragment length polymorphisms (RFLPs). The detection of RFLPs relies on a specialized hybridization procedure called Southern blotting. DNA fragments from digestion of genomic DNA by restriction endonucleases are separated by size electrophoretic-ally, denatured by soaking the agarose gel in alkali, and then blotted onto a nylon membrane to reproduce the distribution of fragments in the gel. The membrane is immersed in a solution containing a radioactively labelled DNA probe. A probe for a sequence that is repeated several times in the human genome generally identifies a few of the thousands of DNA fragments generated when the human genome is digested with a restriction endonuclease. Autoradiography reveals the fragments to which the probe hybridizes. The genomic DNA sequences used in these tests are generally regions containing repetitive DNA (short sequences repeated thousands of times in tandem), which are common in the genomes of higher eukaryotes. The number of repeated units in these DNA regions varies among individuals (except between identical twins).

With a suitable probe, the pattern of bands produced by DNA fingerprinting is distinctive for each individual. Combining the use of several probes makes the test so selective that it can positively identify a single individual in the entire human population. However, the Southern blot procedure requires relatively fresh DNA samples and larger amounts of DNA than are generally present at a crime scene. RFLP analysis sensitivity is augmented by using PCR to amplify vanishingly small amounts of DNA. This allows investigators to obtain DNA fingerprints from a single hair follicle, a drop of blood, a small semen sample from a rape victim, or samples that might be months or even many years old.

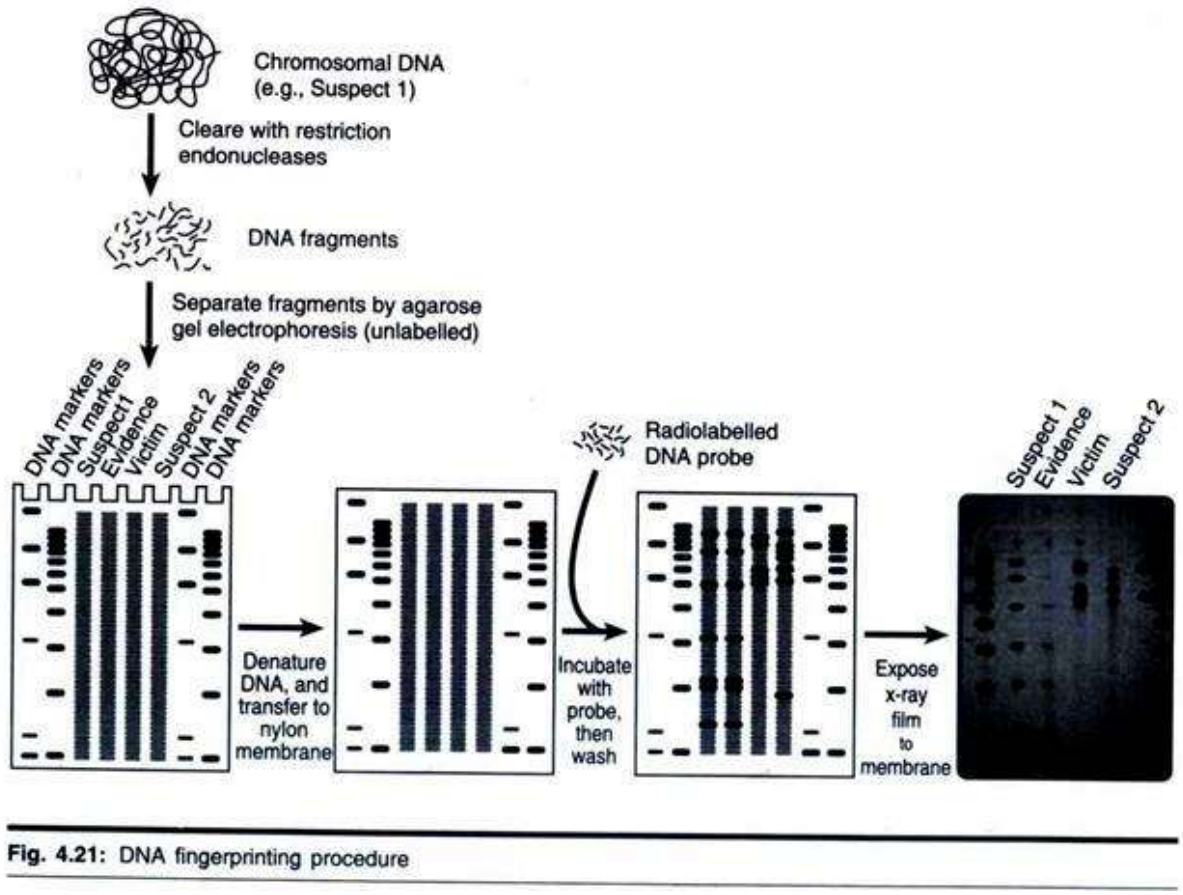


Fig. 4.21: DNA fingerprinting procedure

These methods are proving decisive in court cases worldwide. In the example in Fig. 4.21, the DNA from a semen sample obtained from a rape and murder victim was compared with DNA samples from the victim and two suspects. Each sample was cleaved into fragments and separated by gel electrophoresis. Radioactive DNA probes were used to identify a small subset of fragments that contained sequences complementary to the probe.

The sizes of the identified fragments varied from one individual to the next, as seen here in the different patterns for the three individuals (victim and two suspects) tested. One suspect's DNA exhibits a banding pattern identical to that of a semen sample taken from the victim. This test used a single probe, but three or four different probes would be used (in separate experiments) to achieve an unambiguous positive identification. Such results have been used to both convict and acquit suspects and, in other cases, to establish paternity with an extraordinary degree of certainty. The impact of these procedures on court cases will continue to grow as societies agree on the standards and as formal methods become widely established in forensic laboratories. Even decades-old murder mysteries can be solved: in 1996, DNA fingerprinting helped to confirm the identification of the bones of the last Russian czar and his family, who were assassinated in 1918.

Meaning:

DNA of an individual carries some specific sequence of bases, which do not carry any information for protein synthesis. Such nucleotide base sequences are repeated many times and are found in many places throughout the length of DNA. The number of repeats is very specific in each individual. The tandem repeats of short sequences are called ‘mini satellites’ or ‘variable number tandem repeats’ (VNTRs). Such repeats are used as genetic markers in personal identity.

Technique*:*

1. The first step is to obtain DNA sample of the individual in question.
2. DNA is also isolated from bloodstains, semen stains or hair root from the body of the victim or from victim’s cloth even after many hours of any criminal offence. Even it can be obtained from vaginal swabs of rape victims. The amount of DNA needed for developing fingerprints is very small, only a few nanograms.
3. The DNA is digested with a suitable restriction endonuclease enzyme, which cuts them into fragments.
4. The fragments are subjected to gel electrophoresis by which the fragments are separated according to their size.
5. The separated fragments are copied onto a nitrocellulose filter membrane by Southern blotting technique.
6. Special DNA probes are prepared in the laboratory and made radioactive by labeling with radioactive isotopes. These probes contain repeated sequences of bases complimentary to those on mini satellites.
7. The DNA on the nitrocellulose filter membrane is hybridized with the radioactive probes and the free probes are washed off.
8. The bands to which the radioactive probes have been hybridized are detected through autoradiography. This is a technique where an X-ray film is exposed to the nitrocellulose membrane to mark the places where the radioactive DNA probes have bound to the DNA fragments. These places are marked as dark bands when X-ray film is exposed.
9. The dark bands on the X-ray film represent the DNA fingerprints or DNA profiles.
10. Comparison is made between the banding pattern of collected DNA sample and suspected human subject to confirm the criminal with hundred percent accuracy (Fig.5.24).

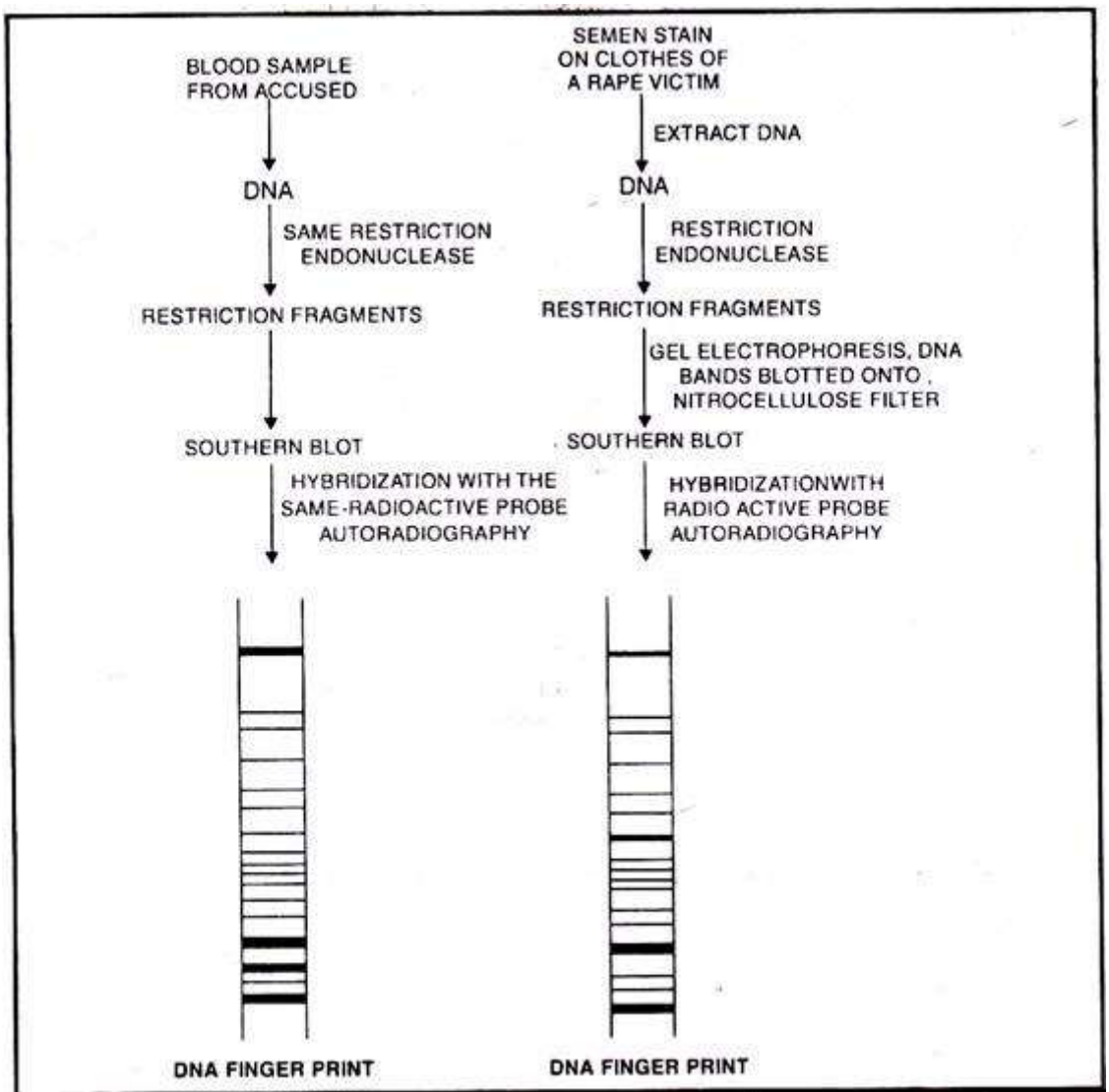


Fig. 5.24 A schematic representation of DNA fingerprinting of a person accused of rape, and of the semen stain recovered from the clothes of rape victim.

Significance:

1. The technique is extensively used as confirmatory test in crime detection in cases of rape and murder.
2. Disputed parentage can be solved by the technique.
3. This method can confirm species of more closeness or far apart from evolutionary point of view so that taxonomical problems can be solved.
4. The technique also can be used to study the breeding pattern of endangered animals.
5. Clinically this method can be used in restoring the health of blood cancer patients.

Y-chromosome analysis

Recent innovations have included the creation of primers targeting polymorphic regions on the Y-chromosome (Y-STR), which allows resolution of a mixed DNA sample from a male and female or cases in which a differential extraction is not possible. Y-chromosomes are

paternally inherited, so Y-STR analysis can help in the identification of paternally related males. Y-STR analysis was performed in the Sally Hemings controversy to determine if Thomas Jefferson had sired a son with one of his slaves. The analysis of the Y-chromosome yields weaker results than autosomal chromosome analysis. The Y male sex-determining chromosome, as it is inherited only by males from their fathers, is almost identical along the patrilineal line. This leads to a less precise analysis than if autosomal chromosomes were testing, because of the random matching that occurs between pairs of chromosomes as zygotes are being made.

Mitochondrial analysis

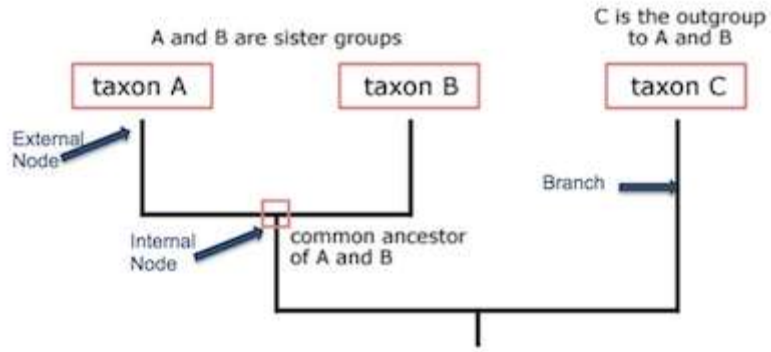
For highly degraded samples, it is sometimes impossible to get a complete profile of the 13 CODIS STRs. In these situations, mitochondrial DNA (mtDNA) is sometimes typed due to there being many copies of mtDNA in a cell, while there may only be 1-2 copies of the nuclear DNA. Forensic scientists amplify the HV1 and HV2 regions of the mtDNA, and then sequence each region and compare single-nucleotide differences to a reference. Because mtDNA is maternally inherited, directly linked maternal relatives can be used as match references, such as one's maternal grandmother's daughter's son. In general, a difference of two or more nucleotides is considered to be an exclusion. Heteroplasmy and poly-C differences may throw off straight sequence comparisons, so some expertise on the part of the analyst is required. mtDNA is useful in determining clear identities, such as those of missing people when a maternally linked relative can be found. mtDNA testing was used in determining that Anna Anderson was not the Russian princess she had claimed to be, Anastasia Romanov. mtDNA can be obtained from such material as hair shafts and old bones/teeth. Control mechanism based on interaction point with data. This can be determined by tooled placement in sample.

Phylogenetic Tree

A phylogenetic tree or **evolutionary tree** is a diagrammatic representation of the evolutionary relationship among various taxa. The phylogenetic tree, including its reconstruction and reliability assessment, is discussed in more detail in Chapter 9. The terms **evolutionary tree**, **phylogenetic tree**, and **cladogram** are often used interchangeably to mean the same thing—that is, the evolutionary relationships among taxa. The term dendrogram is also used interchangeably with cladogram, although there are subtle differences. Thus, it is important to be aware that usage of the vocabulary is not always consistent in the literature, although the context is the same, that is, representation of the evolutionary relationships of taxa.

What Is the Tree of Life?

Hennig's method of visualizing these relationships resulted in what we loosely refer to as a genealogical tree of life. The tree is constructed using a system of **nodes** and **branches**.

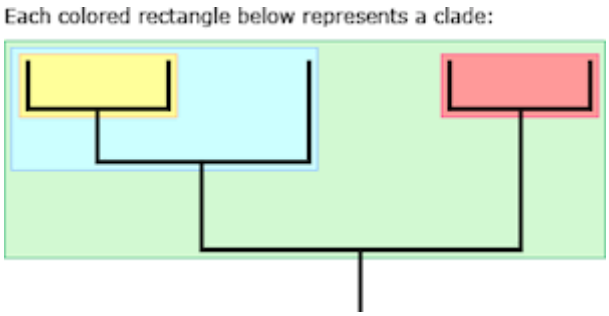


Anatomical description of the parts of a phylogenetic tree

The term **node** refers to any terminating end of a branch (a line). **External nodes** represent the final taxon (singular form of taxa) while **internal nodes** represent a common ancestor that underwent some **speciation event** (where organisms within that taxon stop interbreeding due to reasons like physical isolation, such as the formation of an island, or the preference of a particular physical trait that a subset of the population begins to favor through the process of sexual selection). As a result, speciation events give rise to divergent lineages of taxa and are represented by horizontal branches.

These diverging lines of taxa stem from a common ancestor, resulting in a relationship called **sister taxa** (such as taxon A and taxon B), meaning that they share the closest evolutionary relationship because they stem from the same common ancestor. In this way chimpanzees are our sister taxon, as we are more evolutionarily related to them than we are with, say, gorillas.

Taxa outside of that common ancestor are referred to as **outgroups** as they are more evolutionarily distant in relation than sister taxa are to one another, due to a more distant common ancestor. With each successive speciation event, a new clade is formed within the tree, allowing scientists to identify common ancestors between evolutionarily distant taxa.



Example clades highlighted by color

Anatomy of a phylogenetic tree

When we draw a phylogenetic tree, we are representing our best hypothesis about how a set of species (or other groups) evolved from a common ancestor^11start superscript, 1, end superscript. As we'll explore further in the article on building trees, this hypothesis is based on information we've collected about our set of species – things like their physical features and the DNA sequences of their genes.

[Are phylogenetic trees only for species?]

In a phylogenetic tree, the species or groups of interest are found at the tips of lines referred to as the tree's **branches**. For example, the phylogenetic tree below represents relationships between five species, A, B, C, D, and E, which are positioned at the ends of the branches:

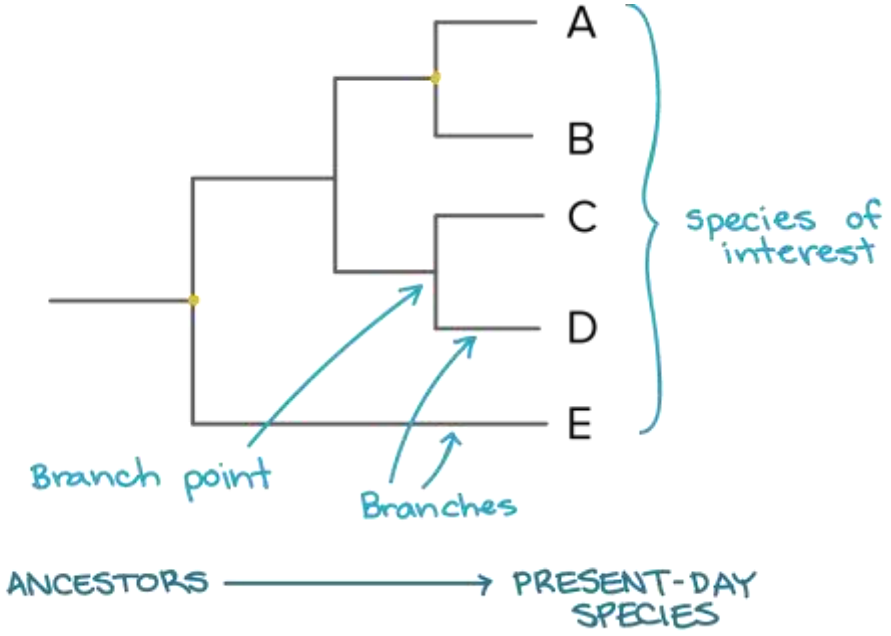
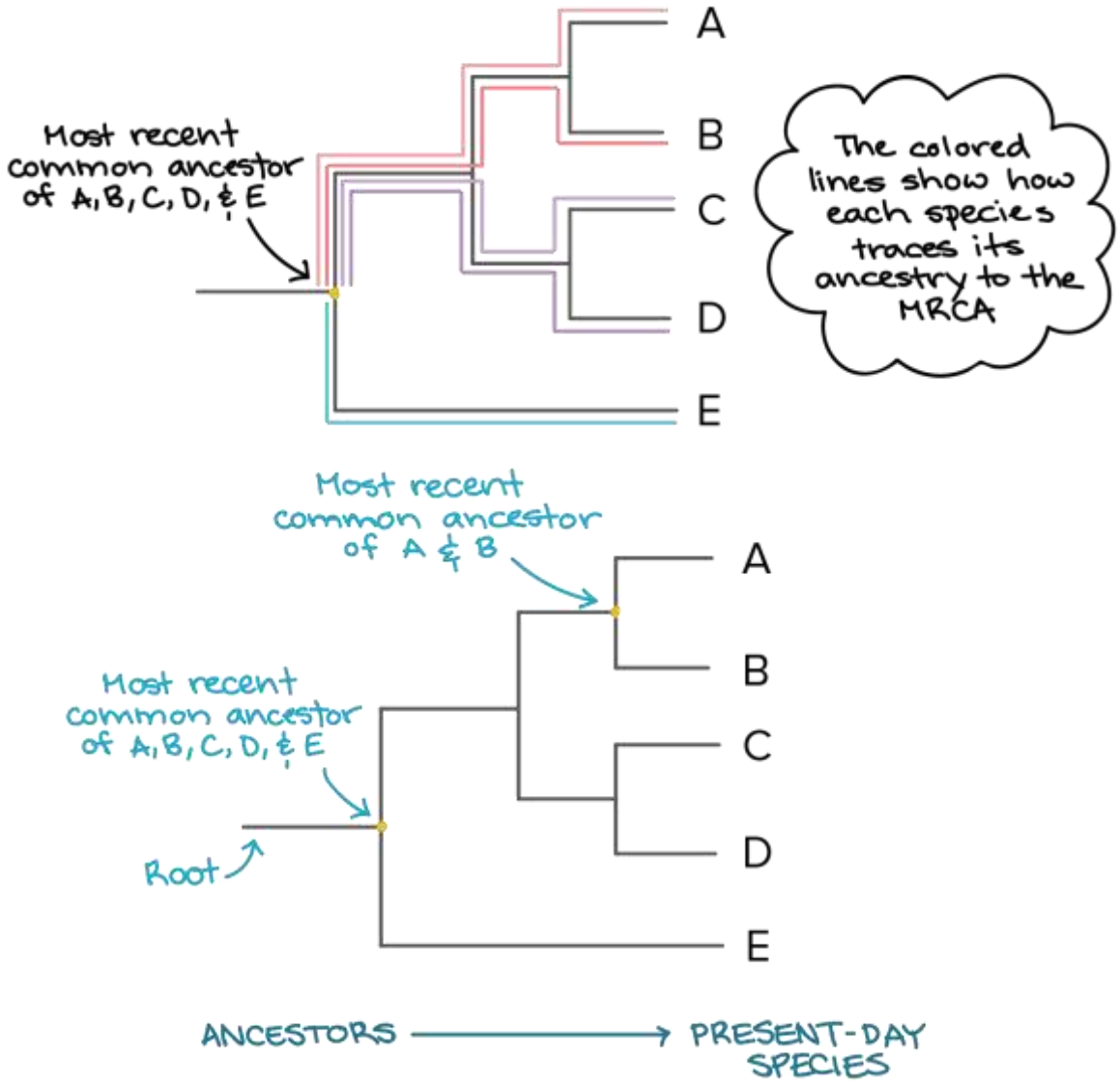


Image modified from *Taxonomy and phylogeny: Figure 2* by Robert Bear et al., CC BY 4.0

The pattern in which the branches connect represents our understanding of how the species in the tree evolved from a series of common ancestors. Each branch point (also called an **internal**

node) represents a **divergence** event, or splitting apart of a single group into two descendant groups.
At each branch point lies the **most recent common ancestor** of all the groups descended from that branch point. For instance, at the branch point giving rise to species A and B, we would find the most recent common ancestor of those two species. At the branch point right above the **root** of the tree, we would find the most recent common ancestor of all the species in the tree (A, B, C, D, E).



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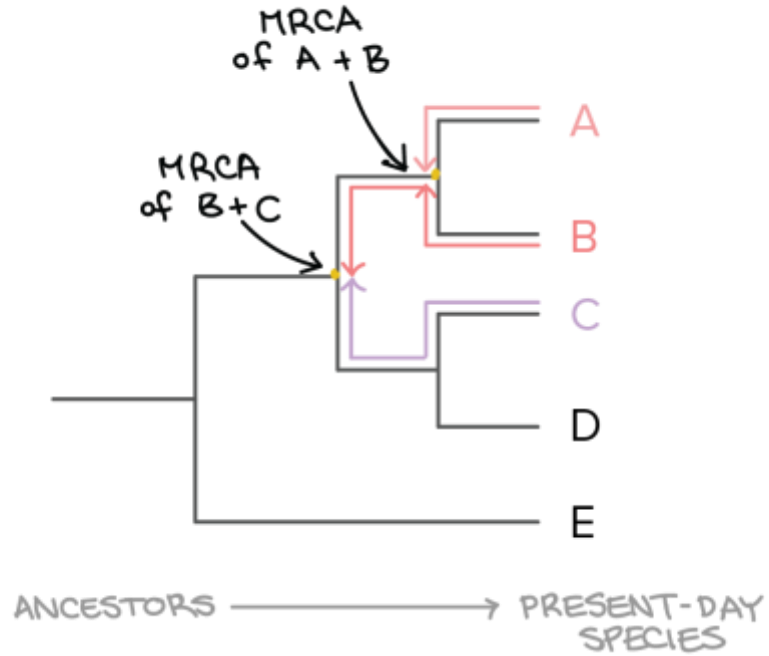
Each horizontal line in our tree represents a series of ancestors, leading up to the species at its end. For instance, the line leading up to species E represents the species' ancestors since it diverged from the other species in the tree. Similarly, the root represents a series of ancestors leading up to the most recent common ancestor of all the species in the tree.

Which species are more related?

In a phylogenetic tree, the **relatedness** of two species has a very specific meaning. Two species are *more* related if they have a *more recent* common ancestor, and *less* related if they have a *less recent* common ancestor. We can use a pretty straightforward method to find the most recent common ancestor of any pair or group of species. In this method, we start at the branch ends carrying the two species of interest and "walk backwards" in the tree until we find the point where the species' lines converge.

For instance, suppose that we wanted to say whether A and B or B and C are more closely related. To do so, we would follow the lines of both pairs of species backward in the tree. Since A and B converge at a common ancestor first as we move backwards, and B only converges with C after its junction point with A, we can say that A and B are more related than B and C.



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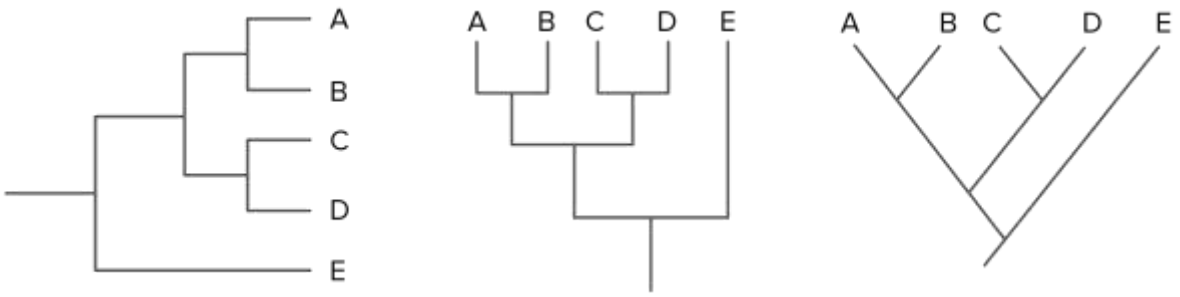
Importantly, there are some species whose relatedness we can't compare using this method. For instance, we can't say whether A and B are more closely related than C and D. That's because, by default, the horizontal axis of the tree doesn't represent time in a direct way. So, we can only compare the timing of branching events that occur on the same lineage (same direct line from the root of the tree), and not those that occur on different lineages.

Some tips for reading phylogenetic trees

You may see phylogenetic trees drawn in many different formats. Some are blocky, like the tree at left below. Others use diagonal lines, like the tree at right below. You may also see trees of either kind oriented vertically or flipped on their sides, as shown for the blocky tree.



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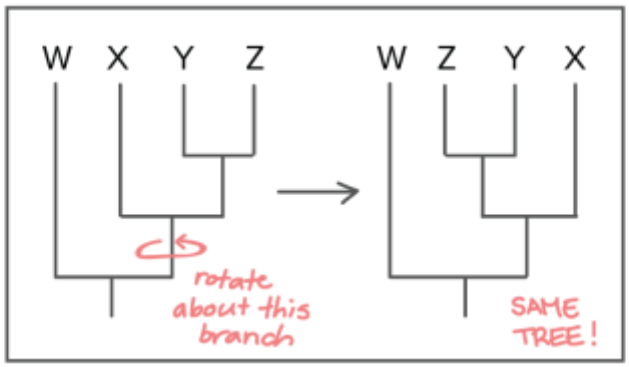
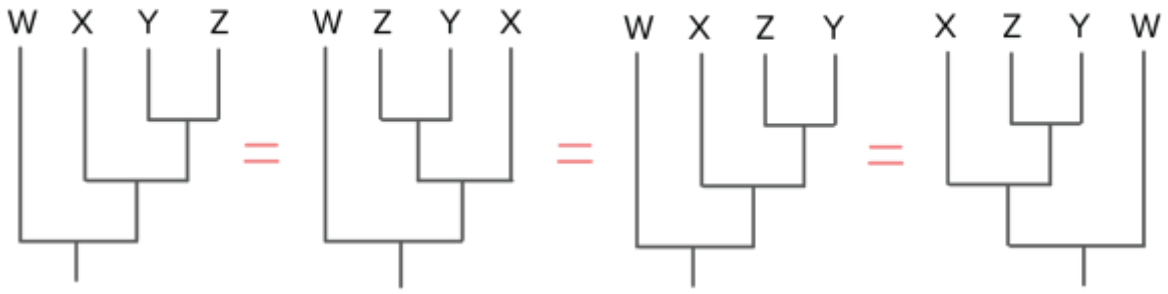
The three trees above represent identical relationships among species A, B, C, D, and E. You may want to take a moment to convince yourself that this is really the case – that is, that no branching patterns or recent-ness of common ancestors are different between the two trees. The identical information in these different-looking trees reminds us that it's the branching pattern (and not the lengths of branches) that's meaningful in a typical tree.

Another critical point about these trees is that if you rotate the structures, using one of the branch points as a pivot, you don't change the relationships. So just like the two trees above, which show the same relationships even though they are formatted differently, all of the trees below show the same relationships among four species:





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If you don't see right away how that is true (and I didn't, on first read!), just concentrate on the relationships and the branch points rather than on the ordering of species (W, X, Y, and Z)

across the tops of the diagrams. That ordering actually doesn't give us useful information. Instead, it's the branch structure of each diagram that tells us what we need to understand the tree.

So far, all the trees we've looked at have had nice, clean branching patterns, with just two lineages (lines of descent) emerging from each branch point. However, you may see trees with a **polytomy** (*poly*, many; *tomy*, cuts), meaning a branch point that has three or more different species coming off of it^22squared. In general, a polytomy shows where we don't have enough information to determine branching order.

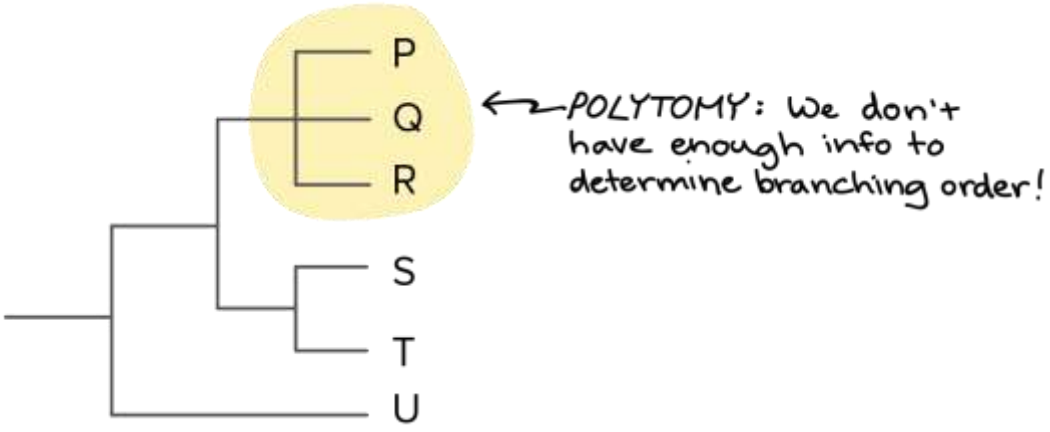


Image modified from *Taxonomy and phylogeny: Figure 2* by Robert Bear et al., CC BY 4.0

If we later get more information about the species in a tree, we may be able to resolve a polytomy using the new information.

Where do these trees come from?

To generate a phylogenetic tree, scientists often compare and analyze many characteristics of the species or other groups involved. These characteristics can include external morphology (shape/appearance), internal anatomy, behaviors, biochemical pathways, DNA and protein sequences, and even the characteristics of fossils.

To build accurate, meaningful trees, biologists will often use many different characteristics (reducing the chances of any one imperfect piece of data leading to a wrong tree). Still, phylogenetic trees are hypotheses, not definitive answers, and they can only be as good as the data available when they're made. Trees are revised and updated over time as new data becomes available and can be added to the analysis. This is particularly true today, as DNA sequencing increases our ability to compare genes between species.

Construction of Phylogenetic tree:

In a phylogenetic tree, the species of interest are shown at the tips of the tree's branches. The branches themselves connect up in a way that represents the evolutionary history of the species—that is, how we think they evolved from a common ancestor through a series of divergence (splitting-in-two) events. At each branch point lies the most recent common ancestor shared by all of the species descended from that branch point. The lines of the tree represent long series of ancestors that extend from one species to the next.

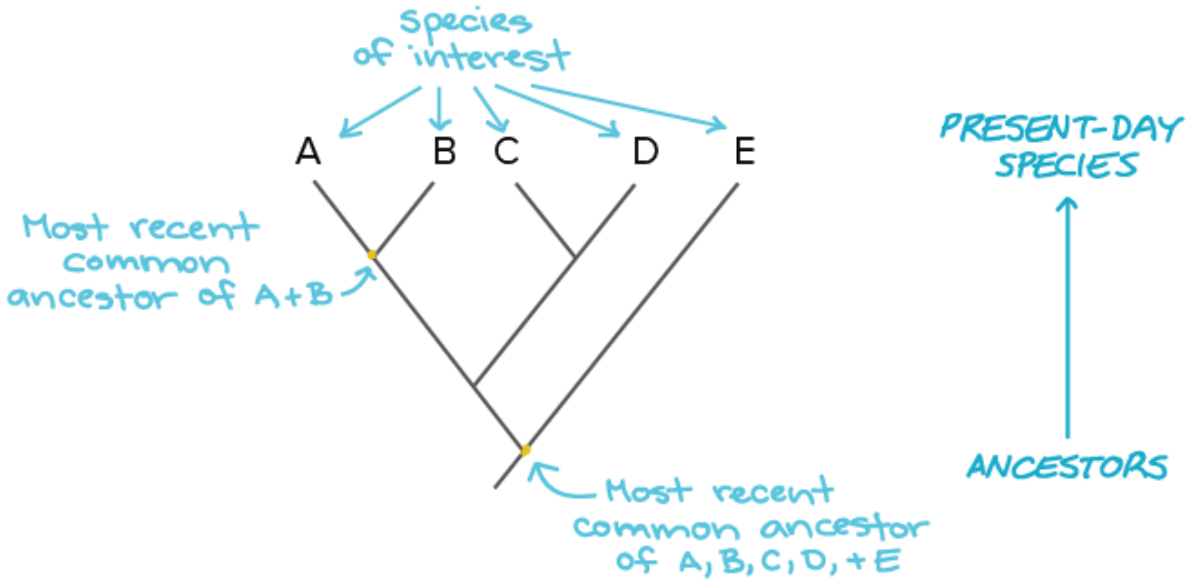
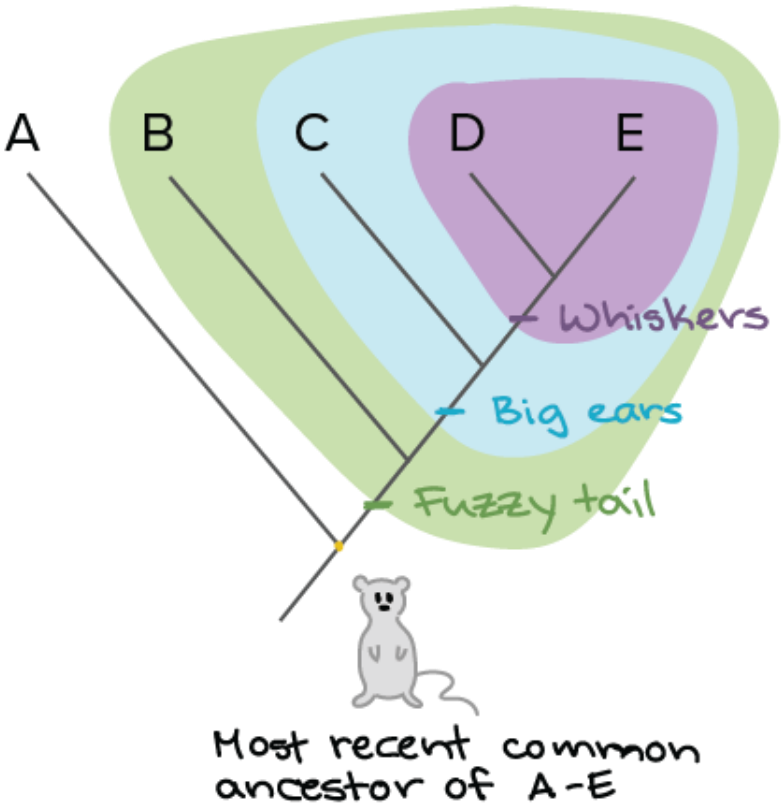


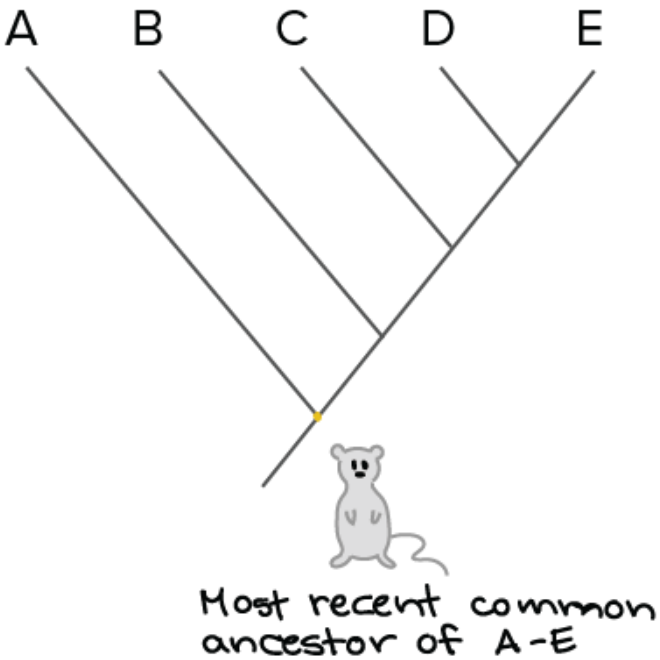
Image modified from Taxonomy and phylogeny: Figure 2, by Robert Bear et al., CC BY 4.0

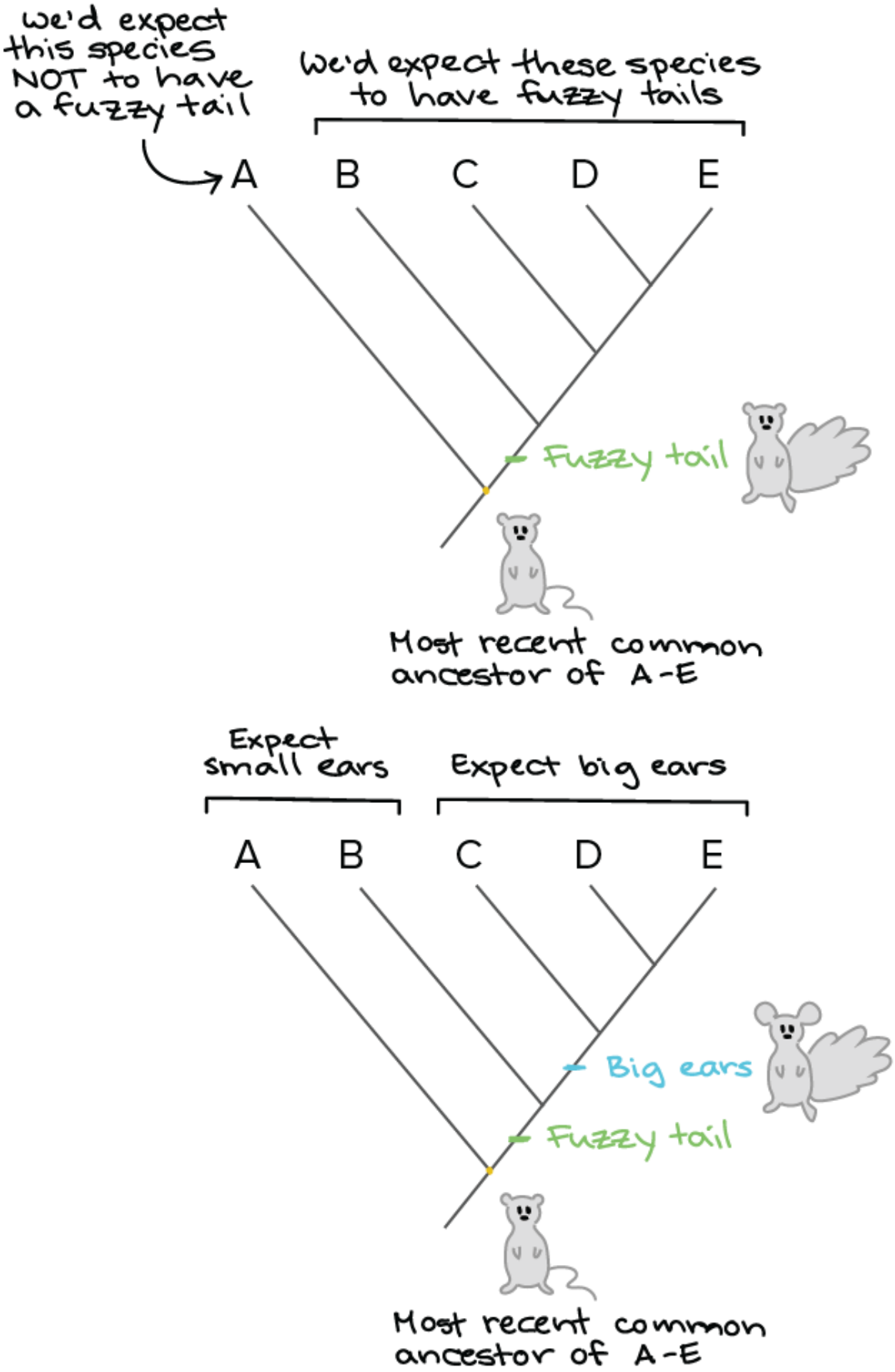
The idea behind tree construction

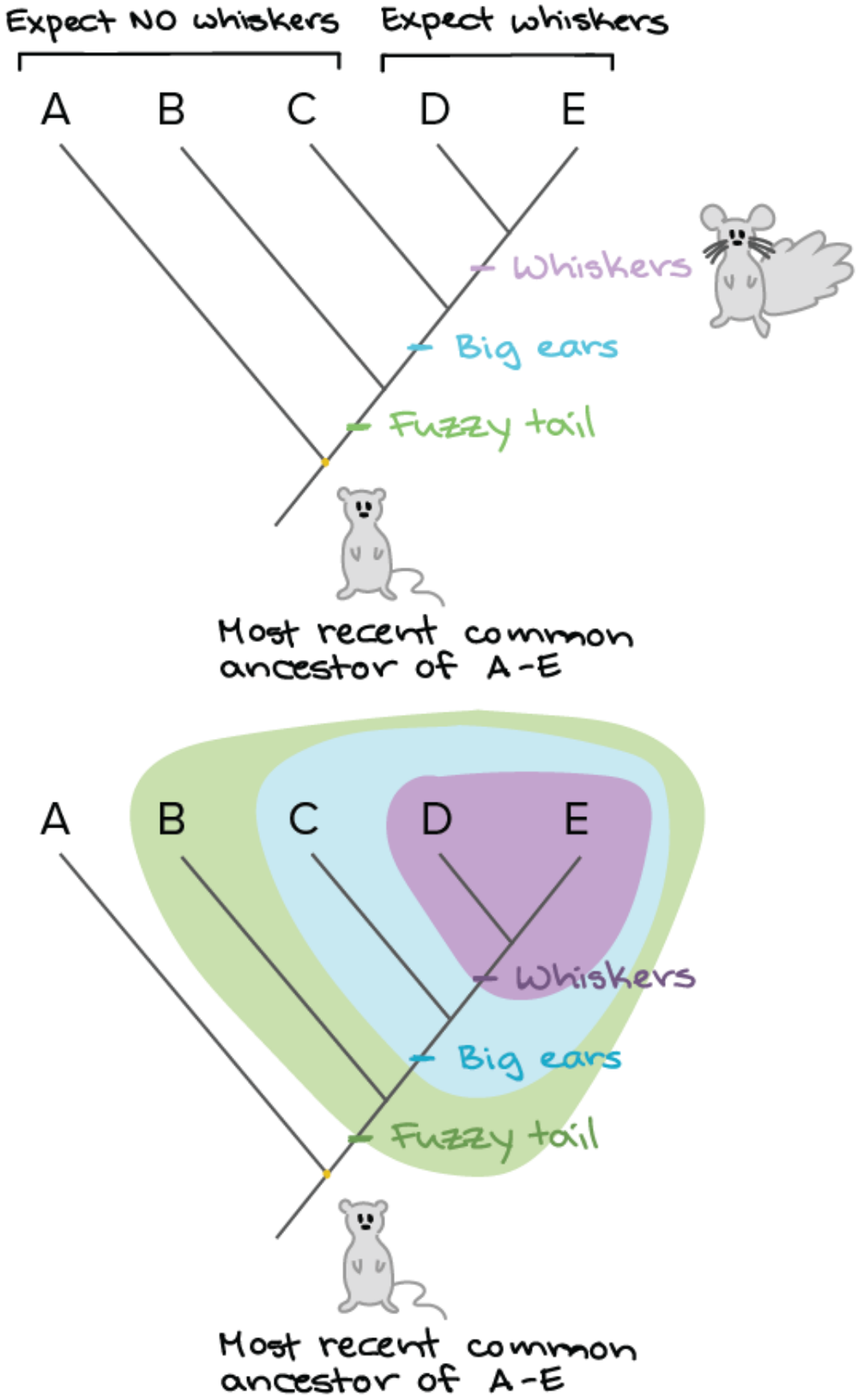
How do we build a phylogenetic tree? The underlying principle is Darwin's idea of "descent with modification." Basically, by looking at the pattern of modifications (novel traits) in present-day organisms, we can figure out—or at least, make hypotheses about—their path of descent from a common ancestor.

As an example, let's consider the phylogenetic tree below (which shows the evolutionary history of a made-up group of mouse-like species). We see three new traits arising at different points during the evolutionary history of the group: a fuzzy tail, big ears, and whiskers. Each new trait is shared by all of the species descended from the ancestor in which the trait arose (shown by the tick marks), but absent from the species that split off before the trait appeared.


A
B
C
D
E
Whiskers
Big ears
Fuzzy tail
Most recent common ancestor of A-E


A
B
C
D
E
Most recent common ancestor of A-E


We'd expect this species NOT to have a fuzzy tail
We'd expect these species to have fuzzy tails
A
B
C
D
E
Fuzzy tail
Most recent common ancestor of A-E
Expect small ears
Expect big ears
A
B
C
D
E
Big ears
Fuzzy tail
Most recent common ancestor of A-E



When we are building phylogenetic trees, traits that arise during the evolution of a group and differ from the traits of the ancestor of the group are called **derived traits**. In our example, a fuzzy tail, big ears, and whiskers are derived traits, while a skinny tail, small ears, and lack of whiskers are **ancestral traits**. An important point is that a derived trait may appear through

either loss or gain of a feature. For instance, if there were another change on the E lineage that resulted in loss of a tail, taillessness would be considered a derived trait.
Derived traits shared among the species or other groups in a dataset are key to helping us build trees. As shown above, shared derived traits tend to form nested patterns that provide information about when branching events occurred in the evolution of the species.
When we are building a phylogenetic tree from a dataset, our goal is to use shared derived traits in present-day species to infer the branching pattern of their evolutionary history. The trick, however, is that we can't watch our species of interest evolving and see when new traits arose in each lineage.
Instead, we have to work backwards. That is, we have to look at our species of interest – such as A, B, C, D, and E – and figure out which traits are ancestral and which are derived. Then, we can use the shared derived traits to organize the species into nested groups like the ones shown above. A tree made in this way is a hypothesis about the evolutionary history of the species – typically, one with the simplest possible branching pattern that can explain their traits.

Example: Building a phylogenetic tree

If we were biologists building a phylogenetic tree as part of our research, we would have to pick which set of organisms to arrange into a tree. We'd also have to choose which characteristics of those organisms to base our tree on (out of their many different physical, behavioural, and biochemical features).
If we're instead building a phylogenetic trees for a class (which is probably more likely for readers of this article), odds are that we'll be given a set of characteristics, often in the form of a table, that we need to convert into a tree. For example, this table shows presence (+) or absence (0) of various features:

Feature	Lamprey	Antelope	Bald eagle	Alligator	Sea bass
Lungs	0	+	+	+	0
Jaws	0	+	+	+	+
Feathers	0	0	+	0	0
Gizzard	0	0	+	+	0
Fur	0	+	0	0	0

Table modified from Taxonomy and phylogeny: Figure 4, by Robert Bear et al., CC BY 4.0

Next, we need to know which form of each characteristic is ancestral and which is derived. For example, is the presence of lungs an ancestral trait, or is it a derived trait? As a reminder, an ancestral trait is what we think was present in the common ancestor of the species of interest. A derived trait is a form that we think arose somewhere on a lineage descended from that ancestor.

Without the ability to look into the past (which would be handy but, alas, impossible), how do we know which traits are ancestral and which derived?

- In the context of homework or a test, the question you are solving may tell you which traits are derived vs. ancestral.
- If you are doing your own research, you may have knowledge that allows you identify ancestral and derived traits (e.g., based on fossils).
- You may be given information about an **outgroup**, a species that's more distantly related to the species of interest than they are to one another.
  If we are given an outgroup, the outgroup can serve as a proxy for the ancestral species. That is, we may be able to assume that its traits represent the ancestral form of each characteristic.
  For instance, in our example (data repeated below for convenience), the lamprey, a jawless fish that lacks a true skeleton, is our outgroup. As shown in the table, the lamprey lacks all of the listed features: it has no lungs, jaws, feathers, gizzard, or fur. Based on this information, we will assume that absence of these features is ancestral, and that presence of each feature is a derived trait.

Feature	Lamprey	Antelope	Bald eagle	Alligator	Sea bass
Lungs	**0**	+	+	+	0
Jaws	**0**	+	+	+	+
Feathers	**0**	0	+	0	0
Gizzard	**0**	0	+	+	0
Fur	**0**	+	0	0	0

Table modified from Taxonomy and phylogeny: Figure 4, by Robert Bear et al., CC BY 4.0

Now, we can start building our tree by grouping organisms according to their shared derived features. A good place to start is by looking for the derived trait that is shared between the largest number of organisms. In this case, that's the presence of jaws: all the organisms except the outgroup species (lamprey) have jaws. So, we can start our tree by drawing the lamprey lineage branching off from the rest of the species, and we can place the appearance of jaws on the branch carrying the non-lamprey species.

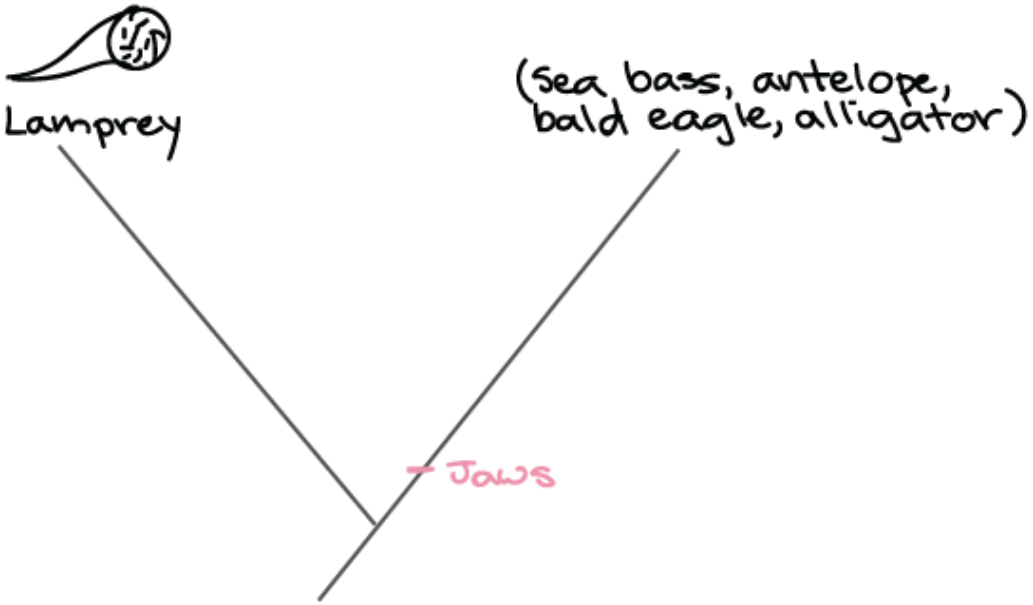


Image based on Taxonomy and phylogeny: Figure 6, by Robert Bear et al., CC BY 4.0

Next, we can look for the derived trait shared by the next-largest group of organisms. This would be lungs, shared by the antelope, bald eagle, and alligator, but not by the sea bass. Based on this pattern, we can draw the lineage of the sea bass branching off, and we can place the appearance of lungs on the lineage leading to the antelope, bald eagle, and alligator.

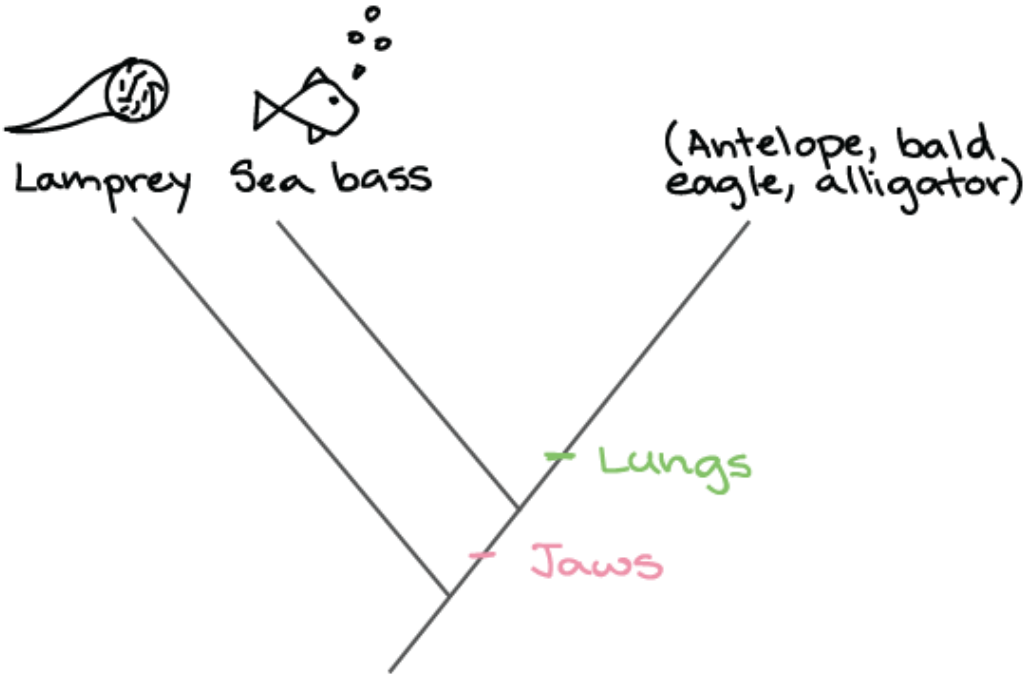


Image based on Taxonomy and phylogeny: Figure 6, by Robert Bear et al., CC BY 4.0

Following the same pattern, we can now look for the derived trait shared by the next-largest number of organisms. That would be the gizzard, which is shared by the alligator and the bald eagle (and absent from the antelope). Based on this data, we can draw the antelope lineage

branching off from the alligator and bald eagle lineage, and place the appearance of the gizzard on the latter.

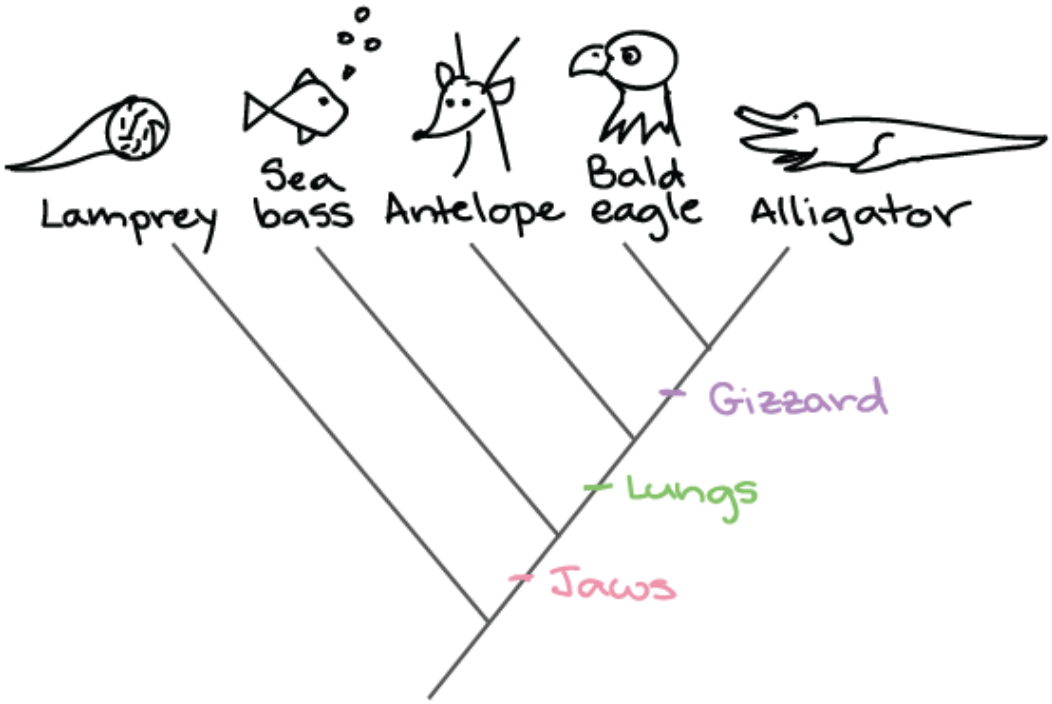


Image based on Taxonomy and phylogeny: Figure 6, by Robert Bear et al., CC BY 4.0

What about our remaining traits of fur and feathers? These traits are derived, but they are not shared, since each is found only in a single species. Derived traits that aren't shared don't help us build a tree, but we can still place them on the tree in their most likely location. For feathers, this is on the lineage leading to the bald eagle (after divergence from the alligator). For fur, this is on the antelope lineage, after its divergence from the alligator and bald eagle.

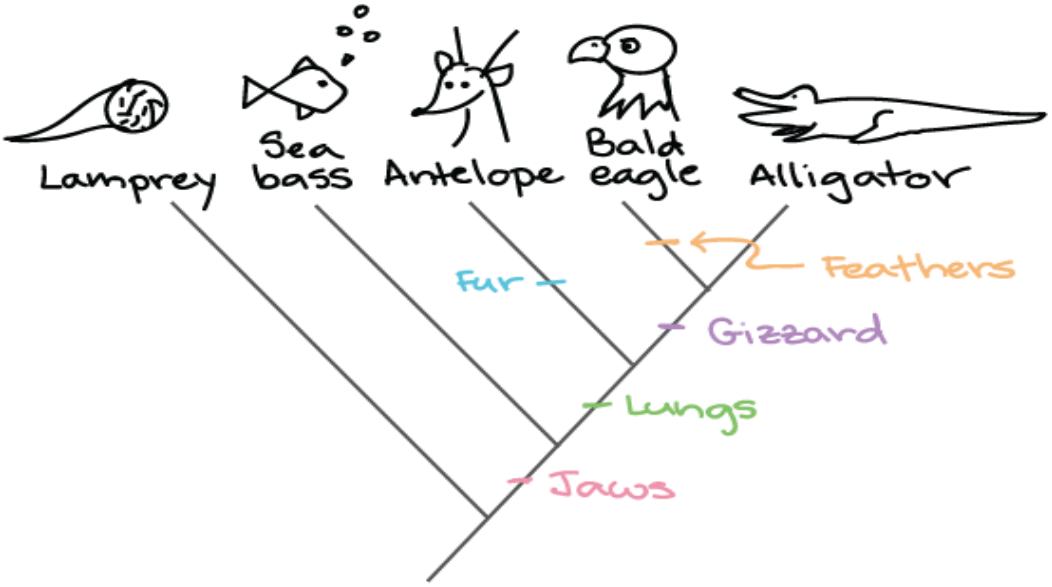


Image based on Taxonomy and phylogeny: Figure 6, by Robert Bear et al., CC BY 4.0

Parsimony and pitfalls in tree construction

When we were building the tree above, we used an approach called **parsimony**. Parsimony essentially means that we are choosing the simplest explanation that can account for our observations. In the context of making a tree, it means that we choose the tree that requires the fewest independent genetic events (appearances or disappearances of traits) to take place.
For example, we *could* have also explained the pattern of traits we saw using the following tree:

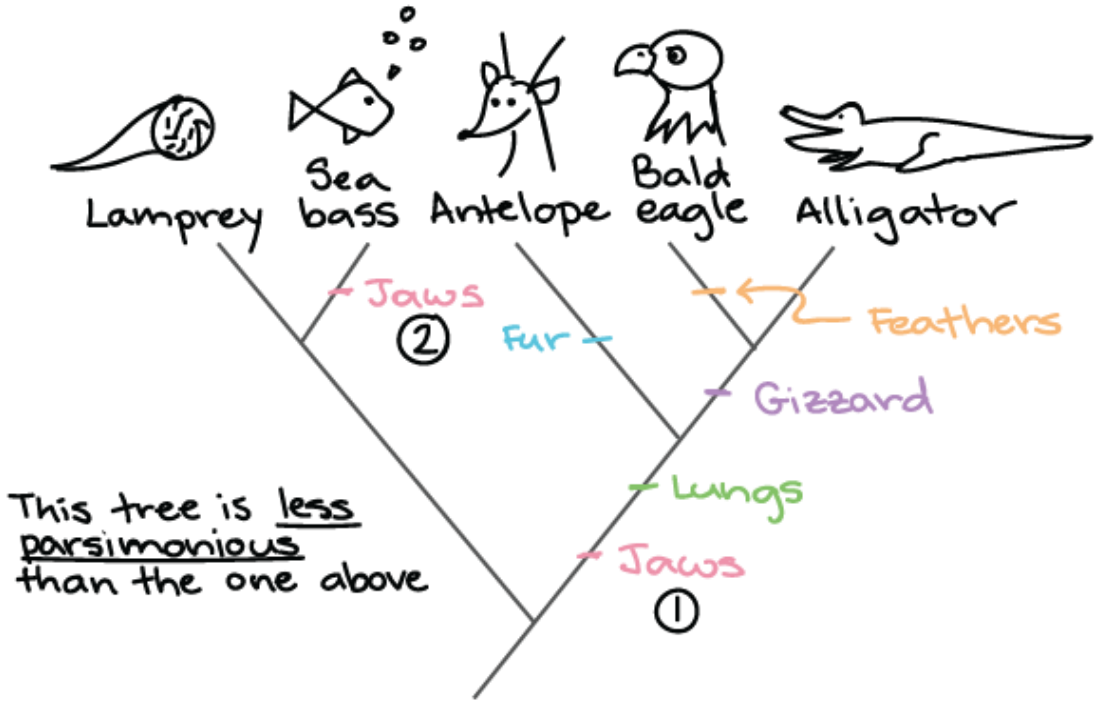


Image based on Taxonomy and phylogeny: Figure 6, by Robert Bear et al., CC BY 4.0

This series of events also provides an evolutionary explanation for the traits we see in the five species. However, it is *less parsimonious* because it requires more independent changes in traits to take place. Because where we've put the sea bass, we have to hypothesize that jaws independently arose two separate times (once in the sea bass lineage, and once in the lineage leading to antelopes, bald eagles, and alligators). This gives the tree a total of 666 tick marks, or trait change events, versus 555 in the more parsimonious tree above.
In this example, it may seem fairly obvious that there is one best tree, and counting up the tick marks may not seem very necessary. However, when researchers make phylogenies as part of their work, they often use a large number of characteristics, and the patterns of these characteristics rarely agree 100\%100%100, percent with one another. Instead, there are some conflicts, where one tree would fit better with the pattern of one trait, while another tree would fit better with the pattern of another trait. In these cases, the researcher can use parsimony to choose the one tree (hypothesis) that fits the data best.
You may be wondering: Why don't the trees all agree with one another, regardless of what characteristics they're built on? After all, the evolution of a group of species did happen in one particular way in the past. The issue is that, when we build a tree, we are reconstructing that evolutionary history from incomplete and sometimes imperfect data. For instance:

- We may not always be able to distinguish features that reflect shared ancestry (**homologous** features) from features that are similar but arose independently (**analogous** features arising by convergent evolution).
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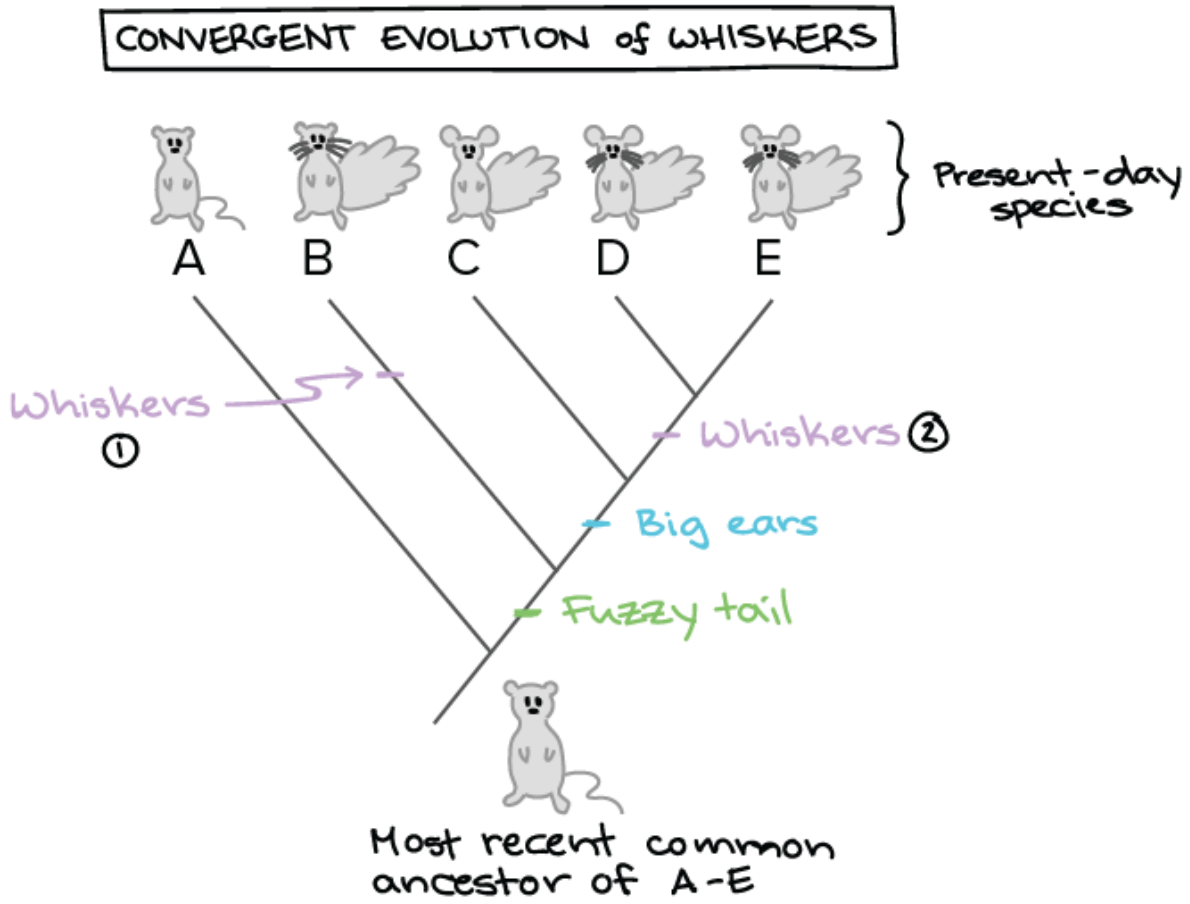
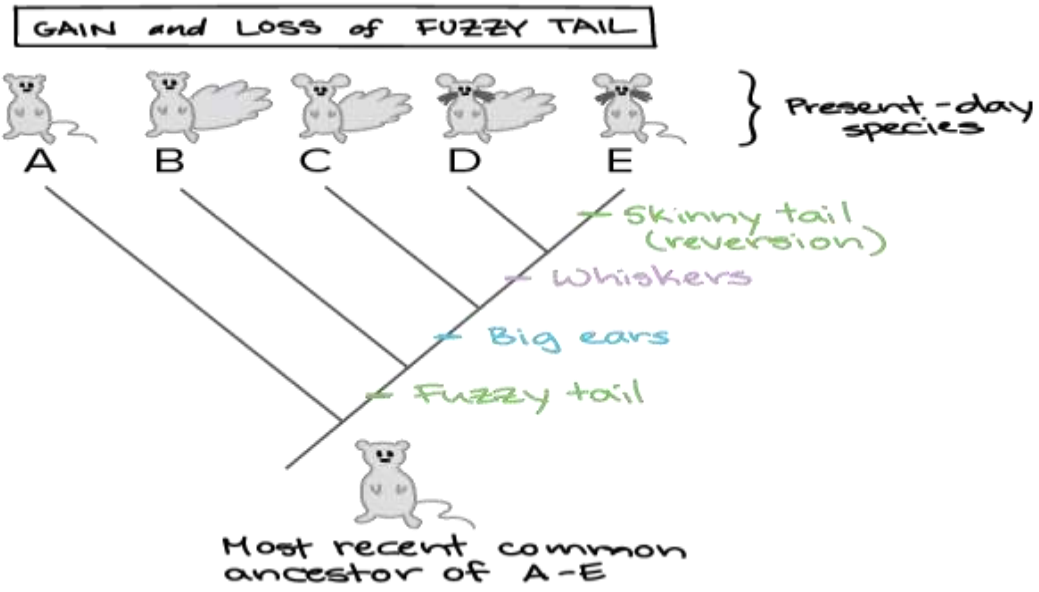


- Traits can be gained and lost multiple times over the evolutionary history of a species. A species may have a derived trait, but then lose that trait (revert back to the ancestral form) over the course of evolution.



Biologists often use many different characteristics to build phylogenetic trees because of sources of error like these. Even when all of the characteristics are carefully chosen and analysed, there is still the potential for some of them to lead to wrong conclusions (because we don't have complete information about events that happened in the past).

Using molecular data to build trees

A tool that has revolutionized, and continues to revolutionize, phylogenetic analysis is DNA sequencing. With DNA sequencing, rather than using physical or behavioural features of organisms to build trees, we can instead compare the sequences of their orthologous (evolutionarily related) genes or proteins.

The basic principle of such a comparison is similar to what we went through above: there's an ancestral form of the DNA or protein sequence, and changes may have occurred in it over evolutionary time. However, a gene or protein doesn't just correspond to a single characteristic that exists in two states.

Instead, each nucleotide of a gene or amino acid of a protein can be viewed as a separate feature, one that can flip to multiple states (e.g., A, T, C, or G for a nucleotide) via mutation. So, a gene with 300300300 nucleotides in it could represent 300300300 different features existing in 444 states! The amount of information we get from sequence comparisons—and thus, the resolution we can expect to get in a phylogenetic tree—is much higher than when we're using physical traits. To analyse sequence data and identify the most probable phylogenetic tree, biologists typically use computer programs and statistical algorithms. In general, though, when we compare the sequences of a gene or protein between species:

- A larger number of differences corresponds to *less* related species
- A smaller number of differences corresponds to *more* related species

For example, suppose we compare the beta chain of haemoglobin (the oxygen-carrying protein in blood) between humans and a variety of other species. If we compare the human and gorilla versions of the protein, we'll find only 111 amino acid difference. If we instead compare the human and dog proteins, we'll find 151515 differences. With human versus chicken, we're up to 454545 amino acid differences, and with human versus lamprey (a jawless fish), we see 127127127 differences. These numbers reflect that, among the species considered, humans are most related to the gorilla and least related to the lamprey.

Probable Questions:

1. what is paralogous gene? Give examples.
2. What is orthologous gene? Give example?
3. What is homologous gene? Give example?
4. Describe the basic steps of DNA Fingerprinting?
5. What is the significance of DNA Fingerprinting?
6. What is the significance of mt DNA analysis?
7. What are the significance of Y chromosome analysis?
8. Define phylogenetic tree?
9. differentiate among cladogram, phenogram, dendogram.
10. What is rooted tree and unrooted tree?
11. How phylogenetic trees are constructed?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
9. Primrose SB and Twyman RM. (2006) Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
10. Sambrook J and Russell D. (2001) Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Pres

ELECTIVE THEORY PAPER (ZET -404)

CYTOGENETICS AND MOLECULAR BIOLOGY

Unit II – Population Genetics

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 404 (CYTOGENETICS AND MOLECULAR BIOLOGY)	V	Inbreeding and heterosis: measurement of inbreeding; panmictic index, inbreeding depression; heterosis; theories of heterosis.	1.0	1	1	
	VI	Genetic structure of populations: Fisher's fundamental theorem of natural selection; genetic variability in natural population; genetic homoeostatis; genetic load and genetic death.		1	1	
	VII	Speciation and evolution at the molecular level: evolution of proteins and nucleotide sequences; regulatory genes and some evolutionary consequences; molecular evolution in the test tube; evolution of genetic systems.		1	1	
	VIII	Gene frequencies and equilibrium: gene frequencies; gene frequencies, gene pool, conservation of gene frequencies.		1	1	

Unit-V

Inbreeding and heterosis: measurement of inbreeding; panmictic index, inbreeding depression; heterosis; theories of heterosis

Objective: In this unit we will discuss about different aspects of inbreeding and heterosis. We will discuss about inbreeding depression, its effect and theories of heterosis. Comparison between hybrid vigour and heterosis will also be discussed in this unit.

Inbreeding:
The process of mating of individuals which are more closely related than the average of the population to which they belong, is called inbreeding. For example, parthenogenesis in animals and apomixes and self-fertilization in plants are the most extreme types of inbreeding.
Inbreeding in self-fertilizing pea plants was a real advantage to Mendel in his studies which provided pure lines of pea plants for his hybridization experiments. The term 'pure line' was coined by W. Johannsen in 1903 for the true breeding, self-fertilized plants.

Methods of Inbreeding:
In plants ova fertilized by the pollen of either the same plants (in case of bisexual plants) or of the other plant of the same genotype (in case of unisexual as well as bisexual plants), is called self-fertilization. However, in bisexual plants numerous structural and functional adaptations have been recorded which help plants with bisexual or hermaphrodite flowers avoid self-fertilization. Normally, inbreeding is affected by restrictions in population size or area which brings about the mating between relatives. Since close relatives have similar genes because of common heritage, inbreeding increases the frequency of homozygotes, but does not bring about a change in overall gene frequencies.
Thus, a mating between two heterozygotes as regards two alleles, A and a will result in half of the population, homozygous for either gene A or a and half of the population heterozygous like the parent but the overall frequencies of A and a remain unchanged:

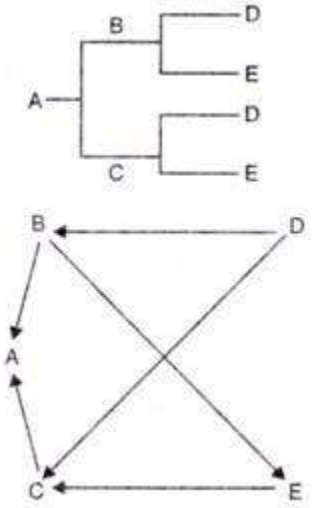
$$Aa \times Aa$$

$$1AA : 1Aa$$

Thus, inbreeding brings about the recessive gene to appear in a homozygous stale (aa). Once a recessive allele is in a homozygous state, natural selection can operate upon the rare recessives. Artificial selection is also possible as the homozygous recessives are phenotypically differentiated from the dominant population.

The inbred pedigrees can be depicted as follows:
Here, B and C are full sibs, i.e., have common parents.

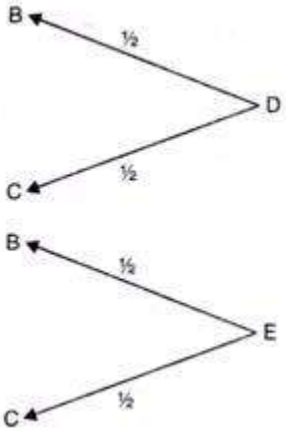
This pedigree can also be represented by the following arrow diagram:



1. Coefficient of Relationship (R):

Coefficient is expression of the amount or degree of any quality possessed by a substance. It is also the degree of physical or chemical change normally occurring in that substance under stated conditions. The coefficient of relationship (R) characterises the percentage of genes held in common by two individuals due to their common ancestry.

Each individual gets only a half of his genotype from one of his parent, each arrow in the above arrow diagram represents a probability of half. The sum (Σ) of all pathways between two individuals through common ancestors is the coefficient of relationship and is represented by R:



(i) R_{BC} = the coefficient of relationship between the full sibs B and C and is calculated as follows:

i.e., individuals B and C contain ½ × ½ = ¼ of their genes in common through ancestor D.

(ii) i.e., individuals B and C contain ½ × ½ = ¼ of their genes in common through ancestor E.

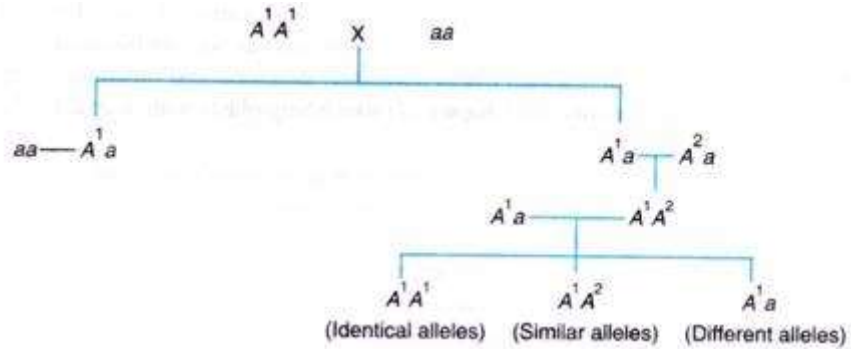
(iii) The sum of these two pathways, the coefficient of relationship, between the full sibs B and C = ¼ + ¼ = ½ = 50 per cent.

2. Inbreeding Coefficient:

In a diploid organism, each gene has two alleles occupying the same locus. They are called identical genes if they have descended from the same gene; such genes are homozygous at the locus.

Such a homozygosity is also caused when two alleles in a diploid organism have not descended from the common gene but the alleles of identical origin are brought together through mating between first cousins. Such alleles are called similar alleles.

The fine difference between these two types of alleles becomes clear by the following chart:



The probability that the two alleles in a zygote are identical by descent, i.e., are the replication product of the same gene of an ancestor is measured by the inbreeding coefficient (F) and is calculated as follows:

1. If the parents B and C are full sibs, i.e., B and C parents are 50 per cent related, the inbreeding coefficient of individual (A) can be calculated by the equation $F_A = ½ R_{BC}$, where R_{BC} is the coefficient of relationship between the full sib parents (B and C) of A.

2. If the common ancestors are not inbred, the inbreeding coefficient is calculated by the equation:

$$F = \Sigma (½)^{n1+n2+1}$$

where n_1, is the number of generations (arrows) from one parent back to the common ancestor and n_2 is the number of generations from the other parent back to the same ancestor.

3. In case the common ancestors are inbred, the inbreeding coefficient is calculated as follows:

$$F = \Sigma (½)^{n1+n2+1(1+Ancestor)}$$

4. The coefficient of inbreeding is also calculated by counting the number of arrows connecting the individual through one parent back to the common ancestor and back again to his other parent by the following equation:

$$F = \Sigma (½)^{n} (1+F_A)$$

n = number of arrows which connect the individual through one parent back to the common ancestor and back again to his other parent. F_A is the inbreeding coefficient of the common

ancestor. For example, the inbreeding coefficient for A in the following arrow diagram can be calculated by following method:
B and C are the parents of A. There is only one pathway from B and C and that goes through ancestor E. Ancestor E is inbred, because its parents (G and H) are full sibs and are 50 per cent related.

The inbreeding coefficient can be calculated as follows:

$F_E = ½ R_{GH}$ (R = the coefficient of relationship between the full sibs G and H)

or $F_E = ½ (0.5) = 0.25$

$F_A = \Sigma (½)^n (1 + F_{E\ (ancestor)})$

or $F_A = (½)^3 (1 + 0.25) = 0.156$

3. Panmixis (Random Mating):

If the breeder assigns no mating restraints upon the selected individuals, their gametes are likely to randomly unite by chance alone. This is commonly the case with outcrossing (non-self-fertilizing) plants. Wind or insect carry pollen from one plant to another in essentially a random manner.
Even livestock such as sheep and range cattle are usually bred panmicticly. The males locate females as they come into heat, copulate with ("cover") and inseminate them without any artificial restrictions as they forage for food over large tracts of grazing land. This mating method is most likely to generate the greatest genetic diversity among the progeny.

4. Assortative and Disassortative Mating:

In sexually reproducing organisms, the most rapid inbreeding system is that between brothers and sisters who share both parents in common. This type of mating is called full-sib mating and produces inbreeding coefficient of 25 per cent in the first generation of inbreeding (F_2 of Mendel).
This rate is reduced in succeeding generations since some of the alleles are now already identical. Within 10 generations, full-sib mating can produce an inbreeding coefficient of 90 per cent. The other inbreeding systems are half-sib mating, parent-offspring mating, third-cousin mating and so on.
All these inbreeding systems are called genetic assortative mating since the parents of each mating type are sorted and mated together on the basis of their genetic relationship. Such a breeding method tends to increase the inbreeding coefficient.
The assortative mating is also of the phenotypic type, i.e., the mating between two like phenotypes, two like dominant phenotypes or between two like recessive phenotypes. If assortative selective mating is continued for many generations, the heterozygotes are eliminated and the resulting population consists of homozygous dominants and homozygous recessives. If more than one locus is considered at a time, the rate of homozygosity achievement will be slower than for one locus. This is so because now the kind of heterozygotes produced will be more combinations of different loci, e.g., Aa BB, AA Bb, …) and eliminating these will need more number of generations. Disassortative mating refers to the mating of unlike phenotypes and genotypes and tends to maintain heterozygosity, as in the case of mating between unlike sexes. This preserves the dissimilarities both genetic as well as phenotypic.

In primitive organism, sexual differences arose at a single gene locus, i.e., one sex was homozygous and the other heterozygous for that locus, and the disassortative mating were the matings between an homozygous and an heterozygous individual for sex locus.
Disassortative mating also results from dichogamy, (Dichogamy = producing mature male and female reproductive structures at different times); self-sterility in plants in which the mating of like phenotypes (inbreeding) is not possible and fertilization between plants with different genotype is favoured. This maintains heterozygosity within a diploid breeding population.

5. Line Breeding:
It is a special form of inbreeding Utilized for the purpose of maintaining a high genetic relationship to a desirable ancestor. D possesses 50 per cent of B's genes and transmits 25 per cent to C. B also contributes 50 per cent of his genes to C. Hence, C contains 50 per cent +25 per cent= 75 per cent B genes and transmits half of them (37.5 per cent) to A. B also contributes 50 per cent of his genes to A. Therefore, A has 50 per cent + 37.5 per cent = 87.5 per cent of B's genes.

Genetic Effects of Inbreeding:
The continuous inbreeding results, genetically, in homozygosity. It produces homozygous stocks of dominant or recessive genes and eliminate heterozygosity from the inbred population. For example, if we start with a population containing 100 heterozygous individuals (Aa) as shown in figure, the expected number of homozygous genotype increasing by 50% due to selfing or inbreeding in each generation. Thus, due to inbreeding in each generation the heterozygosity is reduced by 50% and after 10 generations we can expect the total elimination of heterozygosity from the inbred line and production of two homozygous or pure lines.
But, because a heterozygous individual possesses several heterozygous allelic pairs, we can conclude that inbreeding will operate on all genes loci to produce totally pure or homozygous offspring's. In human beings if inbreeding continued over a number of generations, it would results in increasing homozygosity, but somewhat slowly.

Inbreeding Depression:
In a heterozygote, the inbreeding increases the probability of homozygosity of deleterious recessive alleles in an inbred population. In other words, one of the consequence of inbreeding is a loss in vigour (i.e., less productive vegetatively and reproductively) which commonly accompanies an increase in homozygosity. This is called Inbreeding depression.

Inbreeding depression is found to occur due to following four features of inbreeding:
(1) Increase in frequency of homozygotes,
(2) Increase in variability between different inbred families,
(3) Reduction in value of quantitative character in the direction of recessive values, and
(4) The dependence of this reduction in value upon dominance.
If this inbreeding effect is multiplied for many genes at many loci, there may be a large reduction in value for many traits, including those that affect fitness and survival. In com (maize) for example, E.M. East (1908) and G. H. Shull (1909) studied the effects of inbreeding for 30 generations of inbreeding and found independently, that the yielding ability in these lines

finally reduced to about one-third of the open-pollinated variety from which these samples were derived.

Both of these authors draw the following important conclusions:
(1) A number of lethal and sub-vital types appear in early generations of selfing.
(2) The material rapidly separates into distinct lines, which become increasingly uniform for differences in various morphological and functional characteristics.
(3) Many of the lines decrease in vigour and fecundity until they cannot be maintained even under the most favourable culture conditions.
(4) The lines that survive show a general decline in size and vigour.

Figure 52.4 shows the decline in size and vigour due to inbreeding in maize; here, the inheritance of two quantitative traits namely plant height and grain yield of three lines are shown for 30 generations of inbreeding. It can be noticed that fixation for plant height occurred after five generations of inbreeding. However, yield continued to decline for at least 20 generations until it reached one-third that of open-pollinated variety from which they were derived.

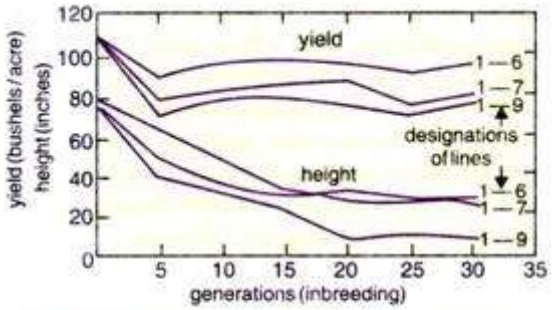


Fig. 52.4. A comparison of three lines of maize, derived from a variety, self-fertilized for 30 generations. Intitially, there were four lines, but it became impossible to maintain one of them beyond 20 generations of inbreeding.

Despite this conspicuous decline, maize was found more tolerant to inbreeding than some organisms where few strains survive two or three generations of inbreeding, e.g., alfalfa and onions. In alfalfa, upon selfing many sub-vital and lethal types appear and the rate of decline of general vigour and productivity is alarming. The very small number of lines which survive give a greatly reduced forage yield.
But onions (a normally cross- pollinated species) are quite tolerant to inbreeding, i.e., they show much less depression in vigour due to inbreeding than alfalfa and maize. Carrot is another cultivated species in which inbreeding leads to loss in vigour and production.
The following cross- pollinated plants are found to be fairly tolerant to inbreeding: sunflowers, rye, timothy, smooth broom-grass and orchard grass. In certain self- pollinated species and normally cross-fertilizing species such as cucurbits, inbreeding is found to be continued indefinitely without any ill effect.

In most animals, inbreeding is found to have less remarkable effects on vigour. For example, in rats continuous brother-sister mating were performed for 25 generations, but no drastic deterioration was detected. In Drosophila, inbreeding usually results in a rapid loss of vigour, but some strains compare favourably with outbreed populations after long continued inbreeding. However, in certain breeds of cattle, intensive inbreeding has led to an unfortunate condition; for example, exhaustive inbreeding and selection of beef cattle breed (Hereford) produced dwarf calves of low economic value.
These calves show characteristic head and body features of the brachycephalic dwarfism (i.e., the characteristic short, broad head, extra long lower jaw, bulging forehead, out of proportion abdomen and short legs). Breeding data indicate that a basic recessive gene is necessary for dwarfing, but additional modifier genes have been postulated to account for the different types of dwarfs.

Practical Applications of Inbreeding:
The correlation of inbreeding and homozygosity exhibits how inbreeding may cause deleterious effects. As we already know that in a heterozygous individual, the harmful recessive alleles remain masked by their normal dominant alleles.
If a heterozygous individual undergoes inbreeding for various generations, there will be equal chances of homozygosity for dominant as well as recessive alleles. In homozygous condition, recessive alleles will be able to express their deleterious phenotypic effects on an individual. On the other hand, the homozygosity for dominant alleles have equal opportunity to express their beneficial phenotypic effects on inbred races.

The practical applications of inbreeding are following:
1. Because inbreeding cause homozygosity of deleterious recessive genes which may result in defective phenotype, therefore, in human society, the religious ethics unknowingly and modem social norms consciously have condemned and banned the marriage of brothers and sisters. Further, the plant breeders and animal breeders too avoid inbreeding's in the individuals due to this reason.

2. The inbreeding because, results in the homozygosity of dominant allele, therefore, it is a best means of mating among hermaphrodites and self-pollinating plant species of several families. The animal breeder have employed the inbreeding to produce best races of horses, dogs, bulls, catties, etc.

The modern race horses, far example, are all descendants of three Arabian stallions imported into England between 1689 and 1730 and mated with several local mares of the slow, heavy type that had carried the medieval knights in heavy armour. The fast runners of F_1 were selected and inbred and stallions of the F_2 appear as beginning points in the pedigrees of almost all modem race horses. This sort of inbreeding in also called line breeding which has been defined as the mating of animals in such a way that their descendants will be kept closely related to an unusually desirable individual.
Similarly, merino sheep are widely known as fine wool producers. They are the result of about 200 years of inbreeding. This strain was being developed in Spain in the 17th century by stock

raisers. They observed that the ancestors of the present day merino sheep had two coats of wool, one composed of long, coarse fibres arising from primary follicles, and a second coat composed of short fine wool arising from clusters of secondary follicles.
Intensive artificial selection was maintained for animals with more uniform production of fine wool and a lesser amount of coarse wool. For a time, Spain had a monopoly on the valuable merino sheep. When France invaded Spain, merino sheep were moved to France where they were maintained and eventually distributed to other parts of the world. Merino sheep were taken to South Africa and in 1796 they were introduced into Australia which has since become the world's largest producer of fine wool.

Heterosis:

When two homozygous inbreeds (a true breeding line obtained by continuous inbreeding) of genetically unlike constituents are crossed together, the resulting hybrids obtained from the crossed seeds are usually robust, vigorous, productive and taller than the either parents.
This increased productivity or superiority over the parents is known as heterosis or hybrid vigour. Heterosis can be defined as the superiority of F_1 hybrid over both the parents in terms of yield or some other character.

History of Heterosis:

Heterosis has been known since the art of hybridization came into existence. Koelreuter (1763) was the first to report hybrid vigour in the hybrids of tobacco, Datura etc. Mendel (1865) observed this in pea crosses.
Darwin (1876) also reported that inbreeding in plants results in deterioration of vigour and the crossing in hybrid vigour. On the basis of his experiments Beal (1877-1882) concluded that F_1 hybrids yield as much as 40 percent more of the parental varieties. From subsequent studies on inter-varietal crosses in maize, it was observed that some of the hybrids show heterosis. While discussing the work on maize during a lecture at Gottingen (West Germany), Dr. G.H. ShuII (1914) proposed the term heterosis (Gr. heteros different and osis = condition). Poweri (1944, 45) reported that the crossing, however, may result in either weak or vigorous hybrids as compared to parental inbreeds.
Hybrid vigour is used as synonym of heterosis. It is generally agreed that hybrid vigour describes only superiority of the hybrid over the parents while heterosis describes the other situation as well i.e., crossing over may result in weak hybrids e.g., many hybrids in tomato are earlier (vegetative phase is replaced by reproductive phase). Earliness in many crops is agriculturally desirable so, it is argued that F, shows faster development in which vegetative phase is replaced by the reproductive phase more quickly than in the parents. On the basis of this explanation it was justified to use the term hybrid vigour as synonym of heterosis.
However, Whaley (1944) was of the opinion that it would be more appropriate to term the developed superiority of the hybrids as hybrid vigour and to refer to the mechanism by which

the superiority is developed as heterosis. Smith (1955) opined that the use of heterosis and hybrid vigour as synonyms is highly desirable on the basis of their long usage.

Types of Heterosis:

Heterosis is of two types:

True heterosis (euheterosis) and pseudo-heterosis.

1. True heterosis:

It is inherited.

It can be further divided into two types:

(a) Mutational true heterosis:

It is the sheltering or shadowing of the deleterious, un-favourable, often lethal, recessive mutant genes by their adaptively superior dominant alleles.

(b) Balanced true heterosis:

It arises out of balanced gene combinations with better adaptive value and agricultural usefulness.

2. Pseudo-heterosis:

Crossing of the two parental forms brings in an accidental, excessive and un-adaptable expression of temporary vigour and vegetative overgrowth. It is also called luxuriance.

Manifestation of Heterosis:

Performance or expression of any character or trait is influenced by many genetic factors — some are positive (stimulating) and others are negative (decreasing). Expressivity of the genes or the degree of manifestation of a character is the result of genetic balance in the action of differently directed factors.

The various manifestations of heterosis may be summarised as follows:

1. Increased Yield:

Increase in yield which may be measured in terms of grain, fruit, seed, leaf, tuber or the whole plant is one of the most important manifestations of heterosis.

2. Increase in Size and General Vigour:

Heterosis results in more vigorous growth which ultimately leads to healthier and faster growing plants with increase in size than the parents.

3. Better Quality:

In many cases heterosis yields better quality which may be accompanied with higher yield.

4. Greater Adaptability:

Hybrids are generally more adapted to environmental changes than the inbred lines due to heterozygosity.

5. More Disease Resistant:

Heterosis sometimes results into development of more disease resistant character in the hybrids.

6. Increased Reproductive Ability:
Hybrids exhibit heterosis by expressing high fertility rate or reproductive ability, which is ultimately expressed in yield character.
7. Increase in Growth Rate:
In many cases the hybrids show faster growth rate than the parents, but that does not always produce larger plant size than the parents.
8. Early Flowering and Maturity:
In many cases the hybrids may show early-ness in flowering and maturity than the parents, for some crops these are the desirable characters for crop improvement. All these manifestations of heterosis can be traced at all levels of hybrid plant organisation.

Heterosis can be observes at different level such as :

a. Molecular Level:
Heterosis is manifested in increased rate of DNA reduplication, transcription and translation influencing the formation of genetic information, enzymatic activity, other regulatory mechanisms and also hybrid protein molecule formation.

b. Functional Level:
Heterosis is expressed as an effective regulation in metabolic processes and morphogenesis in hybrid organism.

c. Cellular Level:
Due to change in electro-kinetic properties of hybrid cell nuclei, the heterosis is manifested by increased mitosis.

d. Organism Level:
Heterosis is expressed as increased growth and differentiation of vegetative organs, synthesis and accumulation of nutritional substances and utilisation of metabolic process for yield formation.

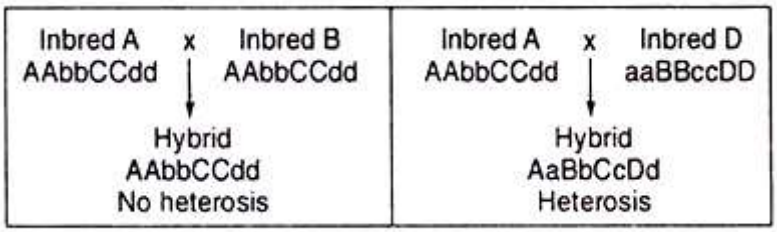
Genetic Basis of Heterosis:
There are two main theories to explain the genetic cause of heterosis.
(A) Dominance Hypothesis:
This hypothesis was proposed by Davenport and further expanded by others. This hypothesis suggests that at each locus dominant allele has the favourable character, whereas the recessive allele has the unfavourable character.
When they are combined together; i.e., in heterozygous condition in the hybrids, the favourable characters get expressed whereas the unfavourable characters are masked. So the heterosis results from the masking of harmful effects of recessive alleles by their dominant alleles.

Dominance Hypothesis has Assumptions:
(a) Dominant genes are beneficial and recessive genes are deleterious.
(b) The loci show addition effects, non-allelic interactions are absent.
(c) No recombination barrier between the genes.

With the help of following example heterosis can be explained:

In a cross between Inbred A (AAbbCCdd) with Inbred B (AAbbCCdd), there will be no heterosis in F_1 hybrid, there is no masking of recessive gene in hybrid. But in another cross, Inbred A (AAbbCCdd) is crossed with Inbred D (aaBBccDD), where the F_1 hybrid is (AaBbCcDd) with all the genes having dominant allele.

As a result the harmful effects of a, b, c, d are hidden by the dominant alleles A, B, C and D. Thus some parents produce heterotic progeny while others do not. Generally parents of diverse or different origin are more likely to produce heterotic progeny than those of similar origin.



Objection:

1. Failure in Isolation of Inbreds as Vigorous as Hybrids:

According to dominance hypothesis it should be possible to get the inbred line with all the dominant genes. Such inbreds should be as vigorous as the F_1 hybrids, but such inbreds have not been isolated.

2. Symmetrical Distribution in F_2:

According to dominance hypothesis, the quantitative characters should not show symmetrical distribution as because dominant and recessive alleles should segregate in the proportion of 3: 1, but generally the F_2 shows symmetrical distribution.

Above two objections can be explained by linked genes. Many of the quantitative characters are governed by linked genes together, so to get the inbred line with all dominant genes require several precisely placed crossovers. In another explanation it can be showed that if the number of genes governing the quantitative characters is large, symmetrical distribution would be obtained even without linkage.

(B) Over-dominance Hypothesis:

This hypothesis was independently proposed by East and Shull. This is sometimes known as single gene heterosis, super-dominance, cumulative action of divergent alleles and stimulation of divergent alleles. According to this hypothesis, heterozygotes are superior to both the homozygotes.

So the heterozygote Aa would be superior to both the homozygotes AA and aa. Consequently, heterozygosity is essential for the cause of heterosis. In case of maize, the gene ma affects maturity. The heterozygote Ma/ma is more vigorous with late maturity than the homozygotes Ma/Ma or ma/ma.

Another proposal by East was that there are several alleles, e.g., a_1, a_2, a_3, a_4…….. etc. with increasingly different functions. Heterozygotes between more divergent alleles would

be more heterotic than those involving less divergent genes, e.g., a_1a_4 is more heterotic than a_1a_2, a_2a_3, a_3a_4, etc. In these cases due to presence of divergent alleles the hybrids have the capacity to perform different functions which is not possible by any of the heterozygotes.

Objection:

1. There are many examples where the superiority is due to the epistatic affect of several non-allelic genes, not due to over-dominance (which is the interaction between allelic genes).

2. There is another objection against over-dominance hypothesis that there are many examples where the homozygotes are superior to the heterozygotes.

Physiological Basis of Heterosis:

Hybrid vigour, the product of heterotic mechanism, is essentially a physiological manifestation.

This better physiological efficiency of hybrids is derived chiefly from:

1. Better initial growth.
2. Greater uptake followed by better utilisation of nutrients by hybrids.

The initial growth activities include the different physiological processes during germination:

(a) Efficient water absorption,
(b) Better activity of enzymes,
(c) Rapid mobilization and utilization of stored food matter,
(d) Transformation and building up of active protoplasmic synthesis.

To explain all these processes different hypotheses have been put forwarded:

1. Initial Capital and Physiological Stimulus:

Large embryo and seed size in hybrids provide initial advantage to the hybrid during germination and early growth of seedlings. This hypothesis is debatable due to two reasons: the greater seedling vigour always not associated with maturity and also hybrid seeds are need not to be always with large size to attain hybrid vigour.

2. Balanced Metabolism and Heterosis at Molecular Level:

The hybrids are endowed with a more balanced metabolism than their inbred parents. Many of the enzymes of heterotic plants exhibit greater efficiency over those of their better parents. The hybrids show better and rapid unfolding of balanced metabolic processes.

3. Mitochondrial Complementation and Heterosis:

ATPase activity of the mixture of mitochondria from different inbred lines of maize sometimes exceed that of the mitochondria of individual lines. This heterotic effect is called as mitochondrial complementation.

The mitochondria of heterotic hybrids absorb more O_2 and have high P/O index, i.e., phosphorylation/oxidation ratio than those of inbred lines and non-heterotic hybrids. This suggests that high level of oxidising phosphorylation and synthesis of high energy ATP bonds

create favourable conditions for biosynthetic processes and important requisite for heterotic development.

4. Greater Ability for uptake and Utilisation of Nutrients:

Heterosis in post germination seedling growth is associated with better absorption and assimilation of several specific substances essential to the fundamental growth processes of the organism; such as nutritional factors, water absorption and other factors.

Efficient uptake and assimilation of nutrients by heterotic hybrid seedlings confer the following advantages:

1. Larger number of leaf primordia.
2. High carboxylase and photophosphorylation activity.
3. Greater leaf area and larger number of leaves.
4. . More branches per panicle and more grains per branch.
5. High grain weight, etc.

Effects or Manifestations of Heterosis:

Whatever may be the cause (genetical or physiological), heterosis is a well known phenomenon.

It is basically the result of the increased metabolic activity of the heterozygote Its effects are well established or manifested in the following three ways:

1. Quantitative Effects:

(a) Increase in size and genetic vigour:

Hybrids are generally more vigorous I;e larger, healthier and faster growing than the parents e.g., head size in cabbage jowar cob size in maize, fruit size in tomato etc.

(b) Increase in yield:

Yield may be measured in terms of grain, fruit, seed, leaf tuber or the whole plant. Hybrids usually have increased yield.

(c) Better quality:

Hybrids show improved quality e.g., hybrids in onion show better keeping quality.

2. Physiological Effects:

(a) Greater resistance to diseases and pests:

Some hybrids show greater resistance to insects or diseases than parents.

(b) Greater flowering and maturity:

Earliness is highly desirable in vegetables In many cases, hybrids are earlier in flowering and maturity than the parents, e.g. tomato hybrids are earlier than their parents.

(c) Greater Adaptability:

Hybrids are usually less susceptible to adverse environmental conditions.

3. Biological Effects:
Hybrids exhibiting heterosis show an increase in biological efficiency i.e., an increase in fertility (reproduction ability) and survival ability.

Heterosis in animals:
(i) Mule is a hybrid from a cross between Jack (Equus hemicus) and Mare (Earns equus) which has been known since ancient times for its well-known qualities of strength and stubbornness.

(ii) Cross between red Sindhi breed of Indian Cattle and Jersey breed of America contams 30% more butter fat in milk.

(iii) Increased pork yield in pigs, more egg laying hens, silk production in silk worms etc.

Crop varieties developed and released by Division of Genetics, IARI during 1991-2001.

Crops	Number of varieties	Names of the varieties/hybrids
Wheat	19	Sonali, Vaishali, HI8381, Kanchan, HP1731, Ganga, HW2004, HP 1761, HP1744, Vidisha, HS365, HI8498, HW1085, Shresth, HD4672, HW2044, HD2733, HI1454, HI1418
Triticale	1	DT46
Rice	9	PNR381, Pusa 44, PNR 162, Pusa 839, Pusa 677, PNR 519, RH-10, Pusa Sugandh-2, Pusa Sugandh-3
Maize	5	PEHM-1, PEHM-2, PEHM-3, Pusa Comp. 3, Pusa Comp. 4
Pearl Millet	6	Pusa 322, Pusa 444, Pusa Bajra 266, Pusa 605, Pusa 415, Pusa 334.
Sorghum	2	RusaChari 121, Pusa chari hybrid 106
Chick pea	7	Pusa 329, Pusa 372, Pusa 362, Pusa 311, Pusa 1003, BGD72, Pusa1053
Pigeon pea	2	Pusa 855, Pusa 9
Mungbean	3	Pusa 9072, Psua 9531, Pusa vishal
Field pea	4	DMR 7, DDR 13, P1542, DDR 23
Lentil	2	Shivalik, Pusa vaibhav
Cow pea	3	Rambha, Rusa safed, Pusa sampada
Mustard	3	Pusa Bahar, Pusa Agrani, Pusa gaurav
Cotton	2	Pusa 8-6, Pusa 31

Comparison between inbreeding depression and hybrid vigour.

1. Increase in Homozygosity vs. Development of Heterozygosity:
Due to inbreeding each line becomes increasingly homozygous, as a consequence the variation within a line decrease rapidly. After 7-8 generations of selfing, the lines becomes almost uniform (99% homozygosity) which are called inbred lines.
Hybridization always favours heterozygosity; the species which reproduce by cross-fertilisation are heterozygous. Due to heterozygosity the effects of many recessive alleles are not expressed in heterosis, only the dominant effects or the multiple effects are expressed.

2. Appearance vs. No/Less Expression of Some Lethal and Sub-Lethal Alleles:
Inbreeding may result in appearance of many harmful characters due to accumulation of harmful recessive alleles after selfing, e.g., chlorophyll deficiency (albina, chlorina), rootless

seedlings, defective floral parts, etc. This type of effects is not found in case of heterosis as most of the lethal characters are expressed in homozygous condition. Heterosis or hybrid vigour prevails heterozygosity, so appearance of such characters does not happen.

3. Reduction vs. Increase in Vigour, Yield and Reproductive Ability:

Due to inbreeding there is a general reduction in vigour of the population, plants become shorter and weaker. The hybrids are generally more vigorous, healthier and increased in size. The reproductive ability also decreases in the population rapidly due to inbreeding, many lines reproduce so poorly that these cannot be maintained. The hybrids exhibiting heterosis show an increase in fertility or reproductive ability.

Inbreeding generally leads to loss in yield; the inbred lines yield much less than the open pollinated varieties from which they are derived. Heterosis is generally expressed as an increase in yield of the hybrid. Commercially this phenomenon is of great importance as an objective of plant breeding.

4. More Susceptible to Disease vs. Increase in Disease Resistance Property:

Due to inbreeding as homozygosity increases, there may be rapid loss of vigour as well as disease resistance property. Whereas due to heterosis the hybrids are known to exhibit a greater resistance to insects or diseases than the parents.

5. Less Adaptable to Changed Environment vs. Greater Adaptability in Hybrids:

The inbred lines are homozygous, so they are less adaptable to changed environment as the modification of characters are not possible according to need. Whereas the hybrids are generally more adaptable to environmental changes than the inbreeds. Variation due to heterozygosity offers the hybrids more adapted to environmental variations.

Probable Questions:

1. Define inbreeding? How it affect genetic diversity?
2. Define inbreeding coefficient? How it can be calculated?
3. Define random mating.
4. Define Assortative and Disassortative Mating.
5. What is inbreeding depression? Why it occurs?
6. What are practical applications of inbreeding.
7. What is heterosis? How it differs from inbreeding depression?
8. Describe types of heterosis?
9. Describe various manifestations of heterosis.
10. Explain two main theories to explain the genetic cause of heterosis.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
9. Primrose SB and Twyman RM. (2006) Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
10. Sambrook J and Russell D. (2001) Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Pres

Unit-VI

Genetic structure of populations: Fisher's fundamental theorem of natural selection; genetic variability in natural population; genetic homoeostatic; genetic load and genetic death

Objective: In this unit we will discuss about Fisher’s fundamental theorem of natural selection. We will also discuss about genetic variability in natural population and genetic homeostasis . We will also discuss about genetic load and genetic death.

Fisher's fundamental theorem of natural selection:

Fisher's fundamental theorem of natural selection is an idea about genetic variance in population genetics developed by the statistician and evolutionary biologist Ronald Fisher. The proper way of applying the abstract mathematics of the theorem to actual biology has been a matter of some debate. It states: "The rate of increase in fitness of any organism at any time is equal to its genetic variance in fitness at that time." Or in more modern terminology: "The rate of increase in the mean fitness of any organism, at any time, that is ascribable to natural selection acting through changes in gene frequencies, is exactly equal to its genetic variance in fitness at that time"

The theorem was first formulated in Fisher's 1930 book The Genetical Theory of Natural Selection. Fisher likened it to the law of entropy in physics, stating that "It is not a little instructive that so similar a law should hold the supreme position among the biological sciences". The model of Quasi-linkage equilibrium was introduced by Motoo Kimura in 1965 as an approximation in the case of weak selection and weak epistasis.

Largely as a result of Fisher's feud with the American geneticist Sewall Wright about adaptive landscapes, the theorem was widely misunderstood to mean that the average fitness of a population would always increase, even though models showed this not to be the case. In 1972, George R. Price showed that Fisher's theorem was indeed correct (and that Fisher's proof was also correct, given a typo or two), but did not find it to be of great significance. The sophistication that Price pointed out, and that had made understanding difficult, is that the theorem gives a formula for part of the change in gene frequency, and not for all of it. This is a part that can be said to be due to natural selection. More recent work builds on Price's understanding in two ways. One aims to improve the theorem by completing it, i.e. by finding a formula for the whole of the change in gene frequency, and accounting for the effects of mutations. The other argues that the partial change is indeed of great conceptual significance, and aims to extend similar partial change results into more and more general population genetic

models. Due to confounding factors, tests of the fundamental theorem are quite rare though Bolnick in 2007 did test this effect in a natural population.

Meaning of Genetic Variation:

Evolution requires genetic variation. If there were no dark moths, the population could not have evolved from mostly light to mostly dark. In order for continuing evolution there must be mechanisms to increase or create genetic variation and mechanisms to decrease it. Mutation is a change in a gene. These changes are the source of new genetic variation. Natural selection operates on this variation.

Genetic variation has two components- allelic diversity and non- random associations of alleles. Alleles are different versions of the same gene. For example, humans can have A, B or O alleles that determine one aspect of their blood type. Most animals, including humans, are diploid—they contain two alleles for every gene at every locus, one inherited from their mother and one inherited from their father.

Locus is the location of a gene on a chromosome. Humans can be AA, AB, AO, BB, BO or OO at the blood group locus. If the two alleles at a locus are the same type (for instance two A alleles) the individual would be called homozygous. An individual with two different alleles at a locus (for example, an AB individual) is called heterozygous. At any locus there can be many different alleles in a population, more alleles than any single organism can possess. For example, no single human can have an A, B and an O allele. Considerable variation is present in natural populations. At 45 percent of loci in plants there is more than one allele in the gene pool. Any given plant is likely to be heterozygous at about 15 percent of its loci. Levels of genetic variation in animals range from roughly 15% of loci having more than one allele (polymorphic) in birds, to over 50% of loci being polymorphic in insects.

Mammals and reptiles are polymorphic at about 20% of their loci – amphibians and fish are polymorphic at around 30% of their loci. In most populations, there are enough loci and enough different alleles that every individual, identical twins excepted, has a unique combination of alleles.

Linkage disequilibrium is a measure of association between alleles of two different genes. If two alleles were found together in organisms more often than would be expected, the alleles are in linkage disequilibrium. If there are two loci in an organism (A and B) and two alleles at each of these loci (A1, A2, B1 and B2), linkage disequilibrium (D) is calculated as D = f (A1B1) * f (A2B2) – f (A1B2) * f (A2B1) (where f(X) is the frequency of X in the population). D varies between -1/4 and 1/4; the greater the deviation from zero, the greater the linkage. The sign is simply a consequence of how the alleles are numbered. Linkage disequilibrium can be the result of physical proximity of the genes. Or, it can be maintained by natural selection if some combinations of alleles work better as a team.

Natural selection maintains the linkage disequilibrium between colour and pattern alleles in *Papilio memnon*. In this moth species, there is a gene that determines wing morphology. One allele at this locus leads to a moth that has a tail; the other allele codes for an untailed moth. There is another gene that determines if the wing is brightly or darkly coloured. There are thus four possible types of moths- brightly coloured moths with and without tails, and dark moths with and without tails. All four can be produced when moths are brought into the lab and bred.

However, only two of these types of moths are found in the wild- brightly coloured moths with tails and darkly colored moths without tails.
The non-random association is maintained by natural selection. Bright, tailed moths mimic the pattern of an unpalatable species. The dark morph is cryptic. The other two combinations are neither mimetic nor cryptic and are quickly eaten by birds. Assortative mating causes a non-random distribution of alleles at a single locus. If there are two alleles (A and a) at a locus with frequencies p and q, the frequency of the three possible genotypes (AA, Aa and aa) will be p^2, 2pq and q^2, respectively. For example, if the frequency of A is 0.9 and the frequency of a is 0.1, the frequencies of AA, Aa and aa individuals will be- 0.81, 0.18 and 0.01. This distribution is called the Hardy-Weinberg equilibrium.
Non-random mating results in a deviation from the Hardy- Weinberg distribution. Humans mate assortatively according to race; we are more likely to mate with someone of own race than another. In populations that mate this way, fewer heterozygotes are found than would be predicted under random mating. A decrease in heterozygotes can be the result of mate choice, or simply the result of population subdivision. Most organisms have a limited dispersal capability, so their mate will be chosen from the local population.
In order for continuing evolution there must be mechanisms to increase or create genetic variation and mechanisms to decrease it. The mechanisms of evolution are mutation, natural selection, genetic drift, recombination and gene flow.

Mechanism of Evolution for Genetic Variation:

Genetic Drift:
Allele frequencies can change due to chances alone. This is called genetic drift. Drift is a binomial sampling error of the gene pool. What this means is, the alleles that form the next generation's gene pool are a sample of the alleles from the current generation. When sampled from a population, the frequency of alleles differs slightly due to chance alone.
Alleles can increase or decrease in frequency due to drift. The average expected change in allele frequency is zero, since increasing or decreasing in frequency is equally probable. A small percentage of alleles may continually change frequency in a single direction for several generations just as flipping a fair coin may, on occasion, result in a string of heads or tails. A very few new mutant alleles can drift to fixation in this manner.
In small populations, the variance in the rate of change of allele frequencies is greater than in large populations. However, the overall rate of genetic drift (measured in substitutions per generation) is independent of population size. If the mutation rate is constant, large and small populations lose alleles to drift at the same rate.
This is because large populations will have more alleles in the gene pool, but they will lose them more slowly. Smaller populations will have fewer alleles, but these will quickly cycle through. This assumes that mutation is constantly adding new alleles to the gene pool and selection is not operating on any of these alleles. Sharp drops in population size can change allele frequencies substantially. When a population crashes, the alleles in the surviving sample may not be representative of the pre-crash gene pool. This change in the gene pool is called the founder effect, because small populations of organisms that invade a new territory (founders) are subject to this.

Many biologists feel the genetic changes brought about by founder effects may contribute to isolated populations developing reproductive isolation from their parent populations. In sufficiently small populations, genetic drift can counteract selection. Mildly deleterious alleles may drift to fixation.

Wright and Fisher disagreed on the importance of drift. Fisher thought populations were sufficiently large that drift could be neglected. Wright argued that populations were often divided into smaller subpopulations. Drift could cause allele frequency differences between subpopulations if gene flow was small enough. If a subpopulation was small enough, the population could even drift through fitness valleys in the adaptive landscape. Then, the subpopulation could climb a larger fitness hill. Gene flow out of this subpopulation could contribute to the population as a whole adapting. This is Wright's Shifting Balance theory of evolution. Both natural selection and genetic drift decrease genetic variation. If they were the only mechanisms of evolution, populations would eventually become homogeneous and further evolution would be impossible. There are, however, mechanisms that replace variation depleted by selection and drift.

Recombination:

Each chromosome in our sperm or egg cells is a mixture of genes from our mother and our father. Recombination can be thought of as gene shuffling. Most organisms have linear chromosomes and their genes lie at specific location (loci) along them. Bacteria have circular chromosomes.

In most sexually reproducing organisms, there is two of each chromosome type in every cell. For instance in humans, every chromosome is paired, one inherited from the mother, the other inherited from the father. When an organism produces gametes, the gametes end up with only one of each chromosome per cell. Haploid gametes are produced from diploid cells by a process called meiosis. In meiosis, homologous chromosomes line up. The DNA of the chromosome is broken on both chromosomes in several places and rejoined with the other strand. Later, the two homologous chromosomes are split into two separate cells that divide and become gametes. But, because of recombination, both of the chromosomes are a mix of alleles from the mother and father. Recombination creates new combinations of alleles. Alleles that arose at different times and different places can be brought together. Recombination can occur not only between genes, but within genes as well. Recombination within a gene can form a new allele. Recombination is a mechanism of evolution because it adds new alleles and combinations of alleles to the gene pool.

Gene Flow:

New organisms may enter a population by migration from another population. If they mate within the population, they can bring new alleles to the local gene pool. This is called gene flow. In some closely related species, fertile hybrids can result from interspecific mating. These hybrids can vector genes from species to species.

Gene flow between more distantly related species occurs infrequently. This is called horizontal transfer. One interesting case of this involves genetic elements called P elements. Margaret Kidwell found that P elements were transferred from some species in the *Drosophila willistoni* group to *Drosophila melanogaster*.

These two species of fruit flies are distantly related and hybrids do not form. Their ranges do, however, overlap. The P elements were vectored into *D. melanogaster* via a parasitic mite that targets both these species. This mite punctures the exoskeleton of the flies and feeds on the "juices". Material, including DNA, from one fly can be transferred to another when the mite feeds. Since P elements actively move in the genome (they are themselves parasites of DNA), one incorporated itself into the genome of a melanogaster fly and subsequently spread through the species. Laboratory stocks of melanogaster caught prior to the 1940's lack of P elements. All natural populations today harbour them.

Evolution within a Lineage for Genetic Variation:

Evolution is a change in the gene pool of a population over time; it can occur due to several factors. Three mechanisms add new alleles to the gene pool- mutation, recombination and gene flow. Two mechanisms remove alleles, genetic drift and natural selection. Drift removes alleles randomly from the gene pool. Selection removes deleterious alleles from the gene pool. The amount of genetic variation found in a population is the balance between the actions of these mechanisms.

Natural selection can also increase the frequency of an allele. Selection that weeds out harmful alleles is called negative selection. Selection that increases the frequency of helpful alleles is called positive, or sometimes positive Darwinian, selection. A new allele can also drift to high frequency. But, since the change in frequency of an allele each generation is random, nobody speaks of positive or negative drift.

Except in rare cases of high gene flow, new alleles enter the gene pool as a single copy. Most new alleles added to the gene pool are lost almost immediately due to drift or selection; only a small percent ever reach a high frequency in the population. Even most moderately beneficial alleles are lost due to drift when they appear. But, a mutation can reappear numerous times.

The fate of any new allele depends a great deal on the organism it appears in. This allele will be linked to the other alleles near it for many generations. A mutant allele can increase in frequency simply because it is linked to a beneficial allele at a nearby locus. This can occur even if the mutant allele is deleterious, although it must not be so deleterious as to offset the benefit of the other allele.

Likewise a potentially beneficial new allele can be eliminated from the gene pool because it was linked to deleterious alleles when it first arose. An allele **"riding on the coat tails"** of a beneficial allele is called a hitchhiker. Eventually, recombination will bring the two loci to linkage equilibrium. But, the more closely linked two alleles are, the longer the hitchhiking will last.

The effects of selection and drift are coupled. Drift is intensified as selection pressures increase. This is because increased selection (i.e. a greater difference in reproductive success among organisms in a population) reduces the effective population size, the number of individuals contributing alleles to the next generation. Adaptation is brought about by cumulative natural selection, the repeated sifting of mutations by natural selection. Small changes, favoured by selection, can be the stepping-stone to further changes. The summation of large numbers of these changes is macroevolution.

Mechanisms that Increase Genetic Variation:

i. Mutation:

The cellular machinery that copies DNA sometimes makes mistakes. These mistakes alter the sequence of a gene. This is called a mutation. There are many kinds of mutations. A point mutation is a mutation in which one "letter" of the genetic code is changed to another. Lengths of DNA can also be deleted or inserted in a gene; these are also mutations. Finally, genes or parts of genes can become inverted or duplicated. Typical rates of mutation are between $10^{-10}$and 10^{-12} mutations per base pair of DNA per generation.

Most mutations are thought to be neutral with regards to fitness. Only a small portion of the genome of eukaryotes contains coding segments. And although some non-coding DNA is involved in gene regulation or other cellular functions, it is probable that most base changes would have no fitness consequence. Most mutations that have any phenotypic effect are deleterious. Mutations that result in amino acid substitutions can change the shape of a protein, potentially changing or eliminating its function. This can lead to inadequacies in biochemical pathways or interfere with the process of development.

Organisms are sufficiently integrated that most random changes will not produce a fitness benefit. Only a very small percentage of mutations are beneficial. The ratio of neutral to deleterious to beneficial mutations is unknown and probably varies with respect to details of the locus in question and environment.

Mutation limits the rate of evolution. The rate of evolution can be expressed in terms of nucleotide substitutions in a lineage per generation. Substitution is the replacement of an allele by another in a population. This is a two-step process- First a mutation occurs in an individual, creating a new allele. This allele subsequently increases in frequency to fixation in the population. The rate of evolution is $k = 2Nvu$ (in diploids) where k is nucleotide substitutions, N is the effective population size, v is the rate of mutation and u is the proportion of mutants that eventually fix in the population.

Mutation need not be limiting over short time spans. The rate of evolution expressed above is given as a steady state equation; it assumes the system is at equilibrium. Given the time frames for a single mutant to fix, it is unclear if populations are ever at equilibrium. A change in environment can cause previously neutral alleles to have selective values; in the short term evolution can run on "stored" variation and thus is independent of mutation rate.

Other mechanisms can also contribute selectable variation. Recombination creates new combinations of alleles (or new alleles) by joining sequences with separate microevolutionary histories within a population. Gene flow can also supply the gene pool with variants. Of course, the ultimate source of these variants is mutation.

ii. Mutant Alleles:

Mutation creates new alleles. Each new allele enters the gene pool as a single copy amongst many. Most are lost from the gene pool, the organism carrying them fails to reproduce, or reproduces but does not pass on that particular allele. A mutant's fate is shared with the genetic background it appears in.

A new allele will initially be linked to other loci in its genetic background, even loci on other chromosomes. If the allele increases in frequency in the population, initially it will be paired with other alleles at that locus—the new allele will primarily be carried in individuals

heterozygous for that locus. The chance of it being paired with itself is low until it reaches intermediate frequency. If the allele is recessive, its effect won't be seen in any individual until a homozygote is formed. The eventual fate of the allele depends on whether it is neutral, deleterious or beneficial.

iii. Neutral Alleles:

Most neutral alleles are lost soon after they appear. The average time (in generations) until loss of a neutral allele is 2(Ne/N) 1n (2N) where N is the effective population size (the number of individuals contributing to the next generation's gene pool) and N is the total population size.

Only a small percentage of alleles fix. Fixation is the process of an allele increasing to a frequency at or near one. The probability of a neutral allele fixing in a population is equal to its frequency. For a new mutant in a diploid population, this frequency is 1/2N.

If mutations are neutral with respect to Fitness, the rate of substitution (k) is equal to the rate of mutation (v). This does not mean every new mutant eventually reaches fixation. Alleles are added to the gene pool by mutation at the same rate they are lost to drift. For neutral alleles that do fix, it takes an average of 4N generations to do so.

However, at equilibrium there are multiple alleles segregating in the population. In small populations, few mutations appear each generation. The ones that fix do so quickly relative to large populations. In large populations, more mutants appear over the generations. But, the ones that fix take much longer to do so. Thus, the rate of neutral evolution (in substitutions per generation) is independent of population size.

The rate of mutation determines the level of heterozygosity at a locus according to the neutral theory. Heterozygosity is simply the proportion of the population that is heterozygous. Equilibrium heterozygosity is given as $H = 4Nv/ [4Nv+1]$ (for diploid populations). H can vary from a very small number to almost one. In small populations, H is small (because the equation is approximately a very small number divided by one). In (biologically unrealistically) large populations, heterozygosity approaches one (because the equation is approximately a large number divided by itself).

Directly testing this model is difficult because N and v can only be estimated for most natural populations. But, heterozygosities are believed to be too low to be described by a strictly neutral model. Solutions offered by neutralists for this discrepancy include hypothesizing that natural populations may not be at equilibrium. At equilibrium there should be a few alleles at intermediate frequency and many at very low frequencies. This is the Ewens- Watterson distribution. New alleles enter a population every generation, most remain at low frequency until they are lost. A few drift to intermediate frequencies, a very few drift all the way to fixation. In *Drosophila pseudoobscura*, the protein Xanthine dehydrogenase (Xdh) has many variants. In a single population, Keith, et. al., found that 59 of 96 proteins were of one type, two others were represented ten and nine times and nine other types were present singly or in low numbers.

iv. Deleterious Alleles:

Deleterious mutants are selected against but remain at low frequency in the gene pool. In diploids, a deleterious recessive mutant may increase in frequency due to drift. Selection cannot

see it when it is masked by a dominant allele. Many disease causing alleles remain at low frequency for this reason.
People who are carriers do not suffer the negative effect of the allele. Unless they mate with another carrier, the allele may simply continue to be passed on. Deleterious alleles also remain in populations at a low frequency due to a balance between recurrent mutation and selection. This is called the mutation load.

v. Beneficial Alleles:
Most new mutants are lost, even beneficial ones. Wright calculated that the probability of fixation of a beneficial allele is 2s. (This assumes a large population size, a small fitness benefit, and that heterozygotes have an intermediate fitness. A benefit of 2s yields an overall rate of evolution- k=4Nvs where v is the mutation rate to beneficial alleles).
An allele that conferred a one percent increase in fitness only has a two percent chance of fixing. The probability of fixation of beneficial type of mutant is boosted by recurrent mutation. The beneficial mutant may be lost several times, but eventually it will arise and stick in a population. (Recall that even deleterious mutants recur in a population.)
Directional selection depletes genetic variation at the selected locus as the fitter allele sweeps to fixation. Sequences linked to the selected allele also increase in frequency due to hitchhiking. The lower the rate of recombination, the larger the window of sequence that hitchhikes. Begun and Aquadro compared the level of nucleotide polymorphism within and between species with the rate of recombination at a locus.
Low levels of nucleotide polymorphism within species coincided with low rates of recombination. This could be explained by molecular mechanisms if recombination itself was mutagenic. In this case, recombination with also be correlated with nucleotide divergence between species. But, the level of sequence divergence did not correlate with the rate of recombination. Thus, they inferred that selection was the cause. The correlation between recombination and nucleotide polymorphism leaves the conclusion that selective sweeps occur often enough to leave an imprint on the level of genetic variation in natural populations.
One example of a beneficial mutation comes from the mosquito *Culex pipiens*. In this organism, a gene that was involved with breaking down organophosphates – common insecticide ingredients -became duplicated. Progeny of the organism with this mutation quickly swept across the worldwide mosquito population.
There are numerous examples of insects developing resistance to chemicals, especially DDT which was once heavily used in this country. And, most importantly, even though “good” mutations happen much less frequently than “bad” ones, organisms with “good” mutations thrive while organisms with “bad” ones die out. If beneficial mutants arise infrequently, the only fitness differences in a population will be due to new deleterious mutants and the deleterious recessives. Selection will simply be weeding out unfit variants. Only occasionally will a beneficial allele be sweeping through a population. The general lack of large fitness differences segregating in natural populations argues that beneficial mutants do indeed arise infrequently. However, the impact of a beneficial mutant on the level of variation at a locus can be large and lasting. It takes many generations for a locus to regain appreciable levels of heterozygosity following a selective sweep.

Mechanisms that Decrease Genetic Variation:

i. Natural Selection:

Some types of organisms within a population leave more offspring than others. Over time, the frequency of the more prolific type will increase. The difference in reproductive capability is called natural selection. Natural selection is the only mechanism of adaptive evolution; it is defined as differential reproductive success of pre-existing classes of genetic variants in the gene pool. The most common action of natural selection is to remove unfit variants as they arise via mutation. In other words, natural selection usually prevents new alleles from increasing its frequency. This led a famous evolutionist, George Williams, to say **"Evolution proceeds in spite of natural selection."**

Natural selection can maintain or deplete genetic variation depending on how it acts. When selection acts to weed out deleterious alleles, or causes an allele to sweep to fixation, it depletes genetic variation. When heterozygotes are fit than either of the homozygotes, however, selection causes genetic variation to be maintained.

This is called balancing selection. An example of this is the maintenance of sickle-cell alleles in human populations subject to malaria. Variation at a single locus determines whether red blood cells are shaped normally or sickled. If a human has two alleles for sickle- cell, he/she develops anaemia — the shape of sickle-cells precludes them carrying normal levels of oxygen. However, heterozygotes that have one copy of the sickle-cell allele, coupled with one normal allele enjoy some resistance to malaria —the shape of sickled cells makes it harder for the plasmodia (malaria causing agents) to enter the cell. Thus, individuals homozygous for the normal allele suffer more malaria than heterozygotes.

Individuals homozygous for the sickle- cell are anaemic. Heterozygotes have the highest fitness of these three types. Heterozygotes pass on both sickle- cell and normal alleles to the next generation. Thus, neither allele can be eliminated from the gene pool. The sickle-cell allele is at its highest frequency in regions of Africa where malaria is most pervasive.

Balancing selection is rare in natural populations. Only a handful of other cases beside the sickle-cell example have been found. At one time population geneticists thought balancing selection could be a general explanation for the levels of genetic variation found in natural populations. That is no longer the case. Balancing selection is only rarely found in natural populations. And, there are theoretical reasons why natural selection cannot maintain polymorphisms at several loci via balancing selection.

Individuals are selected. Dark coloured moths had a higher reproductive success because light coloured moths suffered a higher predation rate. The decline of light coloured alleles was caused by light coloured individuals being removed from the gene pool (selected against). Individual organisms either reproduce or fail to reproduce and are hence the unit of selection.

One way alleles can change in frequency is to be housed in organisms with different reproductive rates. Genes are not the unit of selection (because their success depends on the organism's other genes as well); neither are groups of organisms a unit of selection. There are some exceptions to this "rule" but it is a good generalization.

Organisms do not perform any behaviours that are for the good of their species. An individual organism competes primarily with others of its own species for its reproductive success.

Natural selection favours selfish behaviour because any truly altruistic act increases the recipient's reproductive success while lowering the donors.

Altruists would disappear from a population as the non- altruists would reap the benefits, but not pay the costs, of altruistic acts. Many behaviours appear altruistic. Biologists, however, can demonstrate that these behaviours are only apparently altruistic. Cooperating with or helping other organisms is often the most selfish strategy for an animal. This is called reciprocal altruism. A good example of this is blood sharing in vampire bats. In these bats, those who are lucky enough to find a meal will often share part of it with an unsuccessful bat by regurgitating some blood into the other's mouth.

Biologists have found that these bats form bonds with partners and help each other out when the other is needy. If a bat is found to be a "cheater," his partner will abandon him. The bats are thus not helping each other altruistically; they form pacts that are mutually beneficial.

Helping closely related organisms can appear altruistic; but this is also a selfish behaviour. Reproductive success (fitness) has two components; direct fitness and indirect fitness. Direct fitness is a measure of how many alleles, on average, a genotype contributes to the subsequent generation's gene pool by reproducing. Indirect fitness is a measure of how many alleles identical to its own it helps to enter the gene pool. Direct fitness plus indirect fitness is inclusive fitness. J. B. S. Haldane once remarked he would gladly drown, if by doing so he saved two siblings or eight cousins. Each of his siblings would share one half his alleles; his cousins, one eighth. They could potentially add as many of his alleles to the gene pool as he could.

Natural selection favours traits or behaviours that increase a genotype's inclusive fitness. Closely related organisms share many of the same alleles. In diploid species, siblings share on average at least 50% of their alleles. The percentage is higher if the parents are related. So, helping close relatives to reproduce gets an organism's own alleles better represented in the gene pool.

The benefit of helping relatives increases dramatically in highly inbred species. In some cases, organisms will completely forgo reproducing and only help their relatives reproduce. Ants, and other eusocial insects, have sterile castes that only serve the queen and assist her reproductive efforts. The sterile workers are reproducing by proxy. The words selfish and altruistic have connotations in everyday use that biologists do not intend. Selfish simply means behaving in such a way that one's own inclusive fitness is maximized; altruistic means behaving in such a way that another's fitness is increased at the expense of ones' own. Use of the words selfish and altruistic is not meant to imply that organisms consciously understand their motives.

The opportunity for natural selection to operate does not induce genetic variation to appear — selection only distinguishes between existing variants. Variation is not possible along every imaginable axis, so all possible adaptive solutions are not open to populations. To pick a somewhat ridiculous example, a steel shelled turtle might be an improvement over regular turtles. Turtles are killed quite a bit by cars these days because when confronted with danger, they retreat into their shells — this is not a great strategy against a two ton automobile. However, there is no variation in metal content of shells, so it would not be possible to select for a steel shelled turtle. Here is a second example of natural selection. *Geospiza fortis* lives on the Galapagos islands along with fourteen other finch species. It feeds on the seeds of the plant *Tribulus cistoides*, specializing on the smaller seeds. Another species, *G. Magnirostris*, has a larger beak and specializes on the larger seeds.

The health of these bird populations depends on seed production. Seed production, in turn, depends on the arrival of wet season. In 1977, there was a drought. Rainfall was well below normal and fewer seeds were produced. As the season progressed, the *G. fortis* population depleted the supply of small seeds. Eventually, only larger seeds remained. Most of the finches starved; the population plummeted from about twelve hundred birds to less than two hundred. Peter Grant, who had been studying these finches, noted that larger beaked birds fared better than smaller beaked ones. These larger birds had offspring with correspondingly large beaks. Thus, there was an increase in the proportion of large beaked birds in the population the next generation. To prove that the change in bill size in *Geospiza fortis* was an evolutionary change, Grant had to show that differences in bill size were at least partially genetically based.

He did so by crossing finches of various beak sizes and showing that a finch's beak size was influenced by its parent's genes. Large beaked birds had large beaked offspring; beak size was not due to environmental differences (in parental care, for example).

Natural selection may not lead a population to have the optimal set of traits. In any population, there would be a certain combination of possible alleles that would produce the optimal set of traits (the global optimum); but there are other sets of alleles that would yield a population almost as adapted (local optima). Transition from a local optimum to the global optimum may be hindered or forbidden because the population would have to pass through less adaptive states to make the transition. Natural selection only works to bring populations to the nearest optimal point. This idea is Sewall Wright's adaptive landscape. This is one of the most influential models that shape how evolutionary biologists view evolution.

Natural selection does not have any foresight. It only allows organisms to adapt to their current environment. Structures or behaviours do not evolve for future utility. An organism adapts to its environment at each stage of its evolution. As the environment changes, new traits may be selected for. Large changes in populations are the result of cumulative natural selection. Changes are introduced into the population by mutation; the small minority of these changes that result in a greater reproductive output of their bearers are amplified in frequency by selection. Complex traits must evolve through viable intermediates. For many traits, it initially seems unlikely that intermediates would be viable. Half a wing may be no good for flying, but it may be useful in other ways. Feathers are thought to have evolved as insulation and/or as a way to trap insects.

Later, proto-birds may have learned to glide when leaping from tree to tree. Eventually, the feathers that originally served as insulation now became co-opted for use in flight. A trait's current utility is not always indicative of its past utility. It can evolve for one purpose, and be used later for another. A trait evolved for its current utility is an adaptation; one that evolved for another utility is an exaptation. An example of an exaptation is a penguin's wing. Penguins evolved from flying ancestors; now they are flightless and use their wings for swimming.

ii. Sexual Selection:

In many species, males develop prominent secondary sexual characteristics. A few often cited examples are the peacock's tail; colouring and patterns in male birds in general, voice calls in frogs and flashes in fireflies. Many of these traits are a liability from the standpoint of survival. Any ostentatious trait or noisy, attention getting behaviour will alert predators as well as potential mates. How then could natural selection favour these traits?

Natural selection can be broken down into many components, of which survival is only one. Sexual attractiveness is a very important component of selection, so much so that biologists use the term sexual selection when they talk about this subset of natural selection.
Sexual Selection is natural selection operating on factors that contribute to an organism's mating success. Traits that are a liability to survival can evolve when the sexual attractiveness of a trait outweighs the liability incurred for survival. A male who lives a short time, but produces many offspring is much more successful than a long lived one that produces few.
The former's genes will eventually dominate the gene pool of his species. In many species, especially polygynous species where only a few males monopolize all the females, sexual selection has caused pronounced sexual dimorphism.
In these species males compete against other males for mates. The competition can be either direct or mediated by female choice. In species where females choose, males compete by displaying striking phenotypic characteristics and/or performing elaborate courtship behaviours. The females then mate with the males that most interest them, usually the ones with the most outlandish displays. There are many competing theories as to why females are attracted to these displays.
The good genes model states that the display indicates some component of male fitness. A good genes advocate would say that bright colouring in male birds indicates a lack of parasites. The females are cueing on some signal that is correlated with some other component of viability.
Selection for good genes can be seen in sticklebacks. In these fish, males have red coloration on their sides. Milinski and Bakker showed that intensity of colour was correlated to both parasite load and sexual attractiveness. Females preferred redder males. The redness indicated that he was carrying fewer parasites. Evolution can get stuck in a positive feedback loop. Another model to explain secondary sexual characteristics is called the runaway sexual selection model. R. A. Fisher proposed that females may have an innate preference for some male trait before it appears in a population.
Females would then mate with male carriers when the trait appears. The offspring of these matings have the genes for both the trait and the preference for the trait. As a result, the process snowballs until natural selection brings it into check. Suppose that female birds prefer males with longer than average tail feathers. Mutant males with longer than average feathers will produce more offspring than the short feathered males. In the next generation, average tail length will increase. As the generations progress, feather length will increase because females do not prefer a specific length tail, but a longer than average tail. Eventually tail length will increase to the point where the liability to survival is matched by the sexual attractiveness of the trait and an equilibrium will be established. Note that in many exotic birds male plumage is often very showy and many species do in fact have males with greatly elongated feathers. In some cases these feathers are shed after the breeding season.
None of the above models are mutually exclusive. There are millions of sexually dimorphic species on this planet and the forms of sexual selection probably vary amongst them.

Genetic Load: Genetic load is the difference between the fitness of an average genotype in a population and the fitness of some reference genotype, which may be either the best present in a population, or may be the theoretically optimal genotype. The average individual taken from a population with a low genetic load will generally, when grown in the same conditions,

have more surviving offspring than the average individual from a population with a high genetic load. Genetic load can also be seen as reduced fitness at the population level compared to what the population would have if all individuals had the reference high-fitness genotype. High genetic load may put a population in danger of extinction. Genetic load is a measure of the extent to which the average fitness, viability, or other favourable attribute of a population is decreased by the factor under consideration. Thus there are the following types of load: a mutation load, caused by deleterious mutations; a segregation (or balanced) load, caused by segregation of poor homozygotes at loci where the heterozygote is favoured; a recombination load, caused by the breakup of favourable gene combinations by recombination; a load due to meiotic drive or gamete selection in which these processes produce less favoured genotypes; an incompatibility load, cause by maternal–foetal incompatibility, as in the Rh blood groups; a drift load, caused by unfavourable alleles increasing in frequency by random processes in small populations; and a migration load, caused by immigrants adapted to a different environment.

Causes of genetic load:

Deleterious mutation:

Deleterious mutation load is the main contributing factor to genetic load overall. Most mutations are neutral or slightly deleterious, and occur at a constant rate. The Haldane-Muller theorem of mutation–selection balance says that the load depends only on the deleterious mutation rate and not on the selection coefficient. Specifically, relative to an ideal genotype of fitness 1, the mean population fitness is where U is the total deleterious mutation rate summed over many independent sites. The intuition for the lack of dependence on the selection coefficient is that while a mutation with stronger effects does more harm per generation, its harm is felt for fewer generations.

A slightly deleterious mutation may not stay in mutation–selection balance but may instead become fixed by genetic drift when its selection coefficient is less than one divided by the effective population size. In asexual populations, the stochastic accumulation of mutation load is called Muller's ratchet, and occurs in the absence of beneficial mutations, when after the most-fit genotype has been lost, it cannot be regained by genetic recombination. Deterministic accumulation of mutation load occurs in asexuals when the deleterious mutation rate exceeds one per replication.[8] Sexually reproducing species are expected to have lower genetic loads.[9] This is one hypothesis for the evolutionary advantage of sexual reproduction. Purging of deleterious mutations in sexual populations is facilitated by synergistic epistasis among deleterious mutations. High load can lead to a small population size, which in turn increases the accumulation of mutation load, culminating in extinction via mutational meltdown. The accumulation of deleterious mutations in humans has been of concern to many geneticists.

Beneficial mutation:

New beneficial mutations create fitter genotypes than those previously present in the population. When load is calculated as the difference between the fittest genotype present and the average, this creates a substitutional load. The difference between the theoretical maximum

(which may not actually be present) and the average is known as the "lag load". Motoo Kimura's original argument for the neutral theory of molecular evolution was that if most differences between species were adaptive, this would exceed the speed limit to adaptation set by the substitutional load. However, Kimura's argument confused the lag load with the substitutional load, using the former when it is the latter that in fact sets the maximal rate of evolution by natural selection. More recent "travelling wave" models of rapid adaptation derive a term called the "lead" that is equivalent to the substitutional load, and find that it is a critical determinant of the rate of adaptive evolution.

Inbreeding:

Inbreeding increases homozygosity. In the short run, an increase in inbreeding increases the probability with which offspring get two copies of a recessive deleterious alleles, lowering fitness via inbreeding depression. In a species that habitually inbreeds, e.g. through self-fertilization, recessive deleterious alleles are purged.

Recombination/segregation load:

Combinations of alleles that have evolved to work well together may not work when recombined with a different suite of coevolved alleles, leading to outbreeding depression. Segregation load is the presence of underdominant heterozygotes (i.e. heterozygotes that are less fit than either homozygote). Recombination load arises through unfavourable combinations across multiple loci that appear when favourable linkage disequilibria are broken down. Recombination load can also arise by combining deleterious alleles subject to synergistic epistasis, i.e. whose damage in combination is greater than that predicted from considering them in isolation.

Migration load:

Migration load is the result of nonnative organisms that aren’t adapted to a particular environment coming into that environment. If they breed with individuals who are adapted to that environment, their offspring will not be as fit as they would have been if both of their parents had been adapted to that particular environment. Migration load can also occur in asexually reproducing species, but in this case, purging of low fitness genotypes is more straightforward.

Genetic Death: Permanent non-breeding in a sexually mature individual that is alive but unable (or unwilling) to breed and thus transmit his or her genes to the next generation via the gene pool. Genetic death is fairly rare in most populations of plants and animals, but in developed countries the amount of genetic death amongst humans is increasing, not only in terms of individuals surviving to old age but also because of those who opt to practice birth control. Genetic death is defined as the removal of a gene from the gene pool of a population. It can be the result of infertility, failure to reproduce, or death before sexual maturity of all individuals carrying that gene. Genetic death is not necessarily associated with poor health or loss of life, but rather refers to the impediment of genes being passed on to future generations.

Genetic Death and Evolution:

Genetic death can play a role in evolution by facilitating natural selection. Loss of harmful genes can be beneficial to future generations that are born from that gene pool, potentially increasing fitness. Examples of genetic death constituting a catalyst for evolution include genes can be seen in pathogen resistance, such as the genetic loss of certain cellular receptors that inhibit entry of pathogens into target cells exhibited in genotypes with malaria or AIDS resistance. Olson (1999) describes the concept of gene loss as an engine of evolutionary change. Through natural selection, deleterious mutations of certain genes can be eliminated from the gene pool which can lead to increased fitness. Examples the concept of less is more regarding the propagation of gene loss, include resistance to malaria and AIDs which are both associated with null mutations of specific genes.

Probable questions:

1. Briefly describe Fisher's theorem .
2. What do you mean by genetic variation?
3. How mutation increases genetic variability?
4. How recombination increases genetic variation ?
5. What role gene flow play in increase of genetic variation.
6. What is neutral alleles? How it enhance genetic variation?
7. What is deleterious alleles? How it enhance genetic variation?
8. How sexual selection decrease genetic variation?
9. What is the role of natural selection in decreasing genetic variation?
10. Explain genetic load.
11. Define mutation load.
12. Explain genetic death. How it is related with evolution ?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K

Unit-VII

Speciation and evolution at the molecular level: evolution of proteins and nucleotide sequences; regulatory genes and some evolutionary consequences; molecular evolution in the test tube; evolution of genetic systems

Objective: In this unit we will discuss about evolution of nucleotide and amino acids. We will also discuss about molecular clocks and evolution of genetic systems.

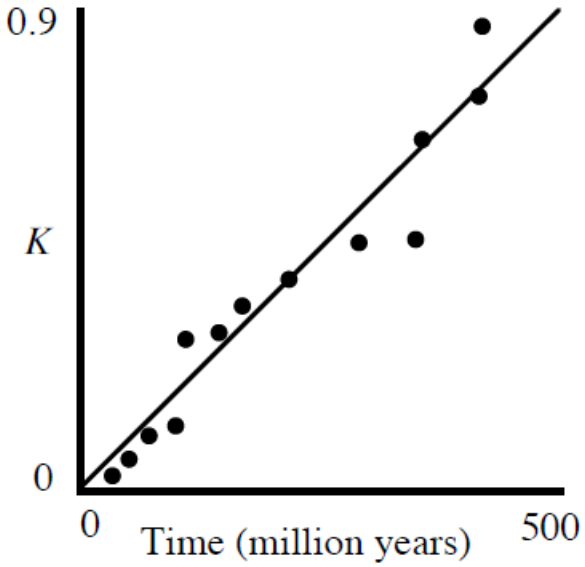
Molecular Evolution:

A. Protein Evolution:

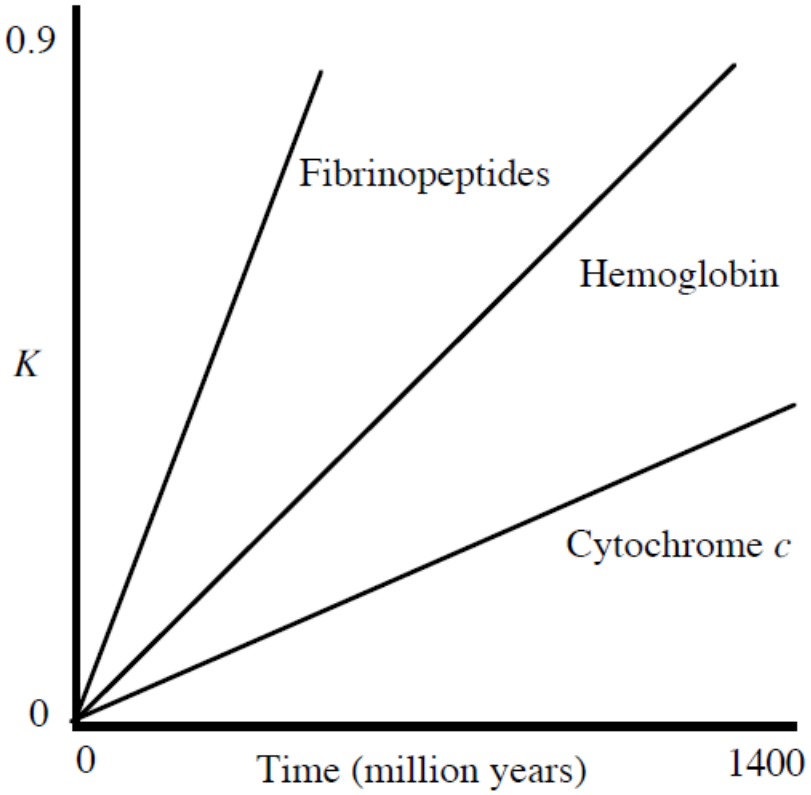
We begin our discussion of molecular evolution with protein evolution for two reasons: First, historically protein sequencing came before DNA sequencing. A method for determining the amino acid sequence of a given protein was developed in mid-1950s. Methods for DNA sequencing were not developed until the mid-1970s. So there was a period of about 20 years where the study of molecular evolution concentrated on protein sequences and this is the period when much of the early molecular evolutionary theory was developed. Today, advances in DNA sequencing have made it much faster and easier to sequence DNA than proteins. Thus the vast majority of protein sequences found in current databases, such as GenBank or SwissProt, were not determined by sequencing the amino acids of the proteins, but instead were inferred from DNA sequences using the universal genetic code. Second, protein evolution is typically 'simpler' than DNA evolution. Although protein sequences are more complex than DNA (20 amino acids versus 4 nucleotides), proteins are generally more conserved throughout evolution and easier to align and compare between distantly related species. As we will see later, DNA is also more complicated because it may be coding or non-coding, and even within coding regions there are synonymous and nonsynonymous nucleotide sites. Here is an example of a partial amino acid (a.a.) sequence of a hypothetical protein: Methionine Proline Valine Serine Threonine Leucine Glycine Isoleucine Lysine Phenylalanine Tryptophan … Met Pro Val Ser Thr Leu Gly Ile Lys Phe Trp M P V S T L G I K F W The 3-letter abbreviation is often useful when indicating coding regions of a DNA sequence, because a codon of 3 nucleotides designates 1 amino acid. The 1-letter abbreviation is most commonly used, particularly in sequence databases. Now lets compare it with the a.a. sequence of the same protein from a different species: M P A S T L G L K F W All proteins begin with Met, so it is usually not considered for comparisons; thus a total of 10 amino acids to compare between species. We can calculate the simple statistic D, the proportion of differences, as: D = 2/10 = 0.20 or 20%. This method is good for sequences that are not too divergent, however, it ignores the possibility of 2 amino acid changes occurring at the same site. For example, what if there was an unobserved, intermediate sequence of: M P G S T L G L K F W Then we really had 3 amino acid replacements instead of 2. This is often called the "multiple hit" problem. Without the intermediate sequence, we do not know if this happened or not, but we can estimate the

probability based on the observed divergence. The expected proportion of differences, K, between 2 sequences is given by the equation: K = -ln (1-D).

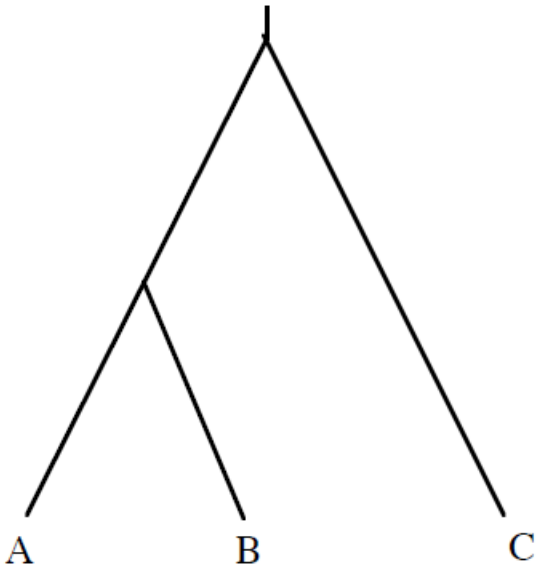
How do we get this? Let r = the rate of amino acid substitution (that is, the proportion of amino acids substituted per unit time, t). The expected proportion of substitutions, K, between 2 sequences is then K = 2rt. The 2 is there because there are 2 lineages on which changes can occur. The proportion of amino acids that have NOT changed over consecutive units of time is (1 – r) 2t The mathematical trick is that (1 – r) 2t ≈ e–2rt, where e is the base of the natural logarithm. The proportion of amino acid that are different is given by, D = 1 - e–2rt = 1 – e-K Taking the natural log of both sides and re-arranging gives us K = -ln (1-D). For our example, K = - ln (1 - 0.20) = 0.22. Note that the difference between D and K becomes larger as D increases. For example, when D = 0.50, K = 0.69. Also, note that this method does not account for substitutions that make previously different amino acids identical, which are assumed to be rare. 2. The Molecular Clock Interestingly, for many proteins the rate of amino acid substitution appears to be constant over long periods of time (where time is estimated from the fossil record). This observation led to the hypothesis of a "molecular clock". For example, when comparing sequences of the blood protein α-globin from various vertebrate species (modified from Hartl and Clark):



The molecular clock does not always tick at the same rate. Although the rate of substitution for a given protein appears to be constant, different proteins may have different rates, for example (modified from Hartl and Clark):



One possible explanation for these patterns is that all of the observed changes in amino acid sequence are neutral. That is, they are neither favoured nor disfavoured by natural selection. Different proteins have different neutral mutation rates, and thus accumulate substitutions at different rates. This hypothesis is the basis of the neutral theory of molecular evolution. An important implication of the molecular clock is that once we know the rate of substitution for a given protein, we can use it to determine the time of divergence of two species for which we have amino acid sequences of that protein. Similarly, if the molecular clock is constant, we can use amino acid sequence divergence to correctly infer phylogenetic relationships among species. It is important to note that the molecular clock is constant with time – not with generations. This is somewhat surprising, given that generation times vary greatly among species and mutations causing changes in protein sequences can occur in each generation. For example, if rodents have a generation time of 1 year and primates have a generation time of 15 years, then we might expect proteins to evolve 15 times faster in rodent lineages. This does not appear to be the case in general (although smaller generation-time effects have been reported). A possible explanation for this is that many amino acid changes may be very slightly deleterious (‘nearly neutral’) and that there is a strong negative correlation between generation time and population size. Thus, more slightly deleterious amino acid changes become fixed due to drift in species with low effective population size (Ne) and long generation times. This may compensate for the reduced fixation rate of neutral mutations due to the smaller number of generations per unit of time. 3. Relative Rate Test Is the molecular clock really constant? A simple and commonly-used way to test this is the relative rate test. For this you need protein sequences from two relatively closely related species and one more distantly related species to be used as an outgroup. Consider the example:



Species A and B should be equally divergent from the outgroup species C. If the molecular clock holds, KAC should equal KBC, and thus KAC – KBC = 0. Two examples (A = mouse, B = rat, C = human) Lactate Dehydrogenase: KAC = 0.804, KBC = 0.803, KAC – KBC = 0.001 Thyroglobulin β: KAC = 0.774, KBC = 0.927, KAC – KBC = -0.153 Here Lactate Dehydrogenase conforms well to the molecular clock, while Thyroglobulin β does not. The significance of the deviation from 0 may be tested computationally using maximum likelihood methods or, alternatively, the number of substitutions along the two branches may be used for a contingency table test, such as a chi-squared test. In general, many proteins appear to evolve in a clock-like manner, although many exceptions have been found in certain proteins and in certain lineages. Thus, there remains much debate about the existence and/or the reliability of the molecular clock. 4. Clock Dispersion If the clock-like evolution observed for many proteins is due only to the random accumulation of neutral amino acid substitutions, then we expect to see a particular statistical distribution of substitutions over time. Specifically, we expect a Poisson distribution, which is a distribution describing the occurrence of rare events. An important feature of the Poisson distribution is that the mean (μ) and variance ($\sigma 2$) are equal. This suggests a test of the molecular clock by testing the ratio of the variance to the mean: R = $\sigma 2$ /μ Under neutrality, R = 1. The value of R has been observed to vary greatly from protein to protein, but in general there is an excess of variance, that is R > 1. For example, in a comparison of 9 mammalian proteins R ranged from 0.16 to 35.6. Some proteins actually appear to follow the clock more regularly than expected (R < 1), while many others show greater dispersion than expected under a neutral model of molecular evolution (R > 1).

Evolution of proteins is studied by comparing the sequences and structures of proteins from many organisms representing distinct evolutionary clades. If the sequences/structures of two proteins are similar indicating that the proteins diverged from a common origin, these proteins are called as homologous proteins. More specifically, homologous proteins that exist in two distinct species are called orthologs. Whereas, homologous proteins encoded by the genome of a single species are called paralogs. The phylogenetic relationships of proteins are examined

by multiple sequence comparisons. Phylogenetic trees of proteins can be established by the comparison of sequence identities among proteins. Such phylogenetic trees have established that the sequence similarities among proteins reflect closely the evolutionary relationships among organisms. Protein evolution describes the changes over time in protein shape, function, and composition. Through quantitative analysis and experimentation, scientists have strived to understand the rate and causes of protein evolution. Using the amino acid sequences of haemoglobin and cytochrome c from multiple species, scientists were able to derive estimations of protein evolution rates. What they found was that the rates were not the same among proteins. Each protein has its own rate, and that rate is constant across phylogenies (i.e., haemoglobin does not evolve at the same rate as cytochrome c, but haemoglobins from humans, mice, etc. do have comparable rates of evolution). Not all regions within a protein mutate at the same rate; functionally important areas mutate more slowly and amino acid substitutions involving similar amino acids occurs more often than dissimilar substitutions. Overall, the level of polymorphisms in proteins seems to be fairly constant. Several species (including humans, fruit flies, and mice) have similar levels of protein polymorphism.

Relation to nucleic acid evolution:

Protein evolution is inescapably tied to changes and selection of DNA polymorphisms and mutations because protein sequences change in response to alterations in the DNA sequence. Amino acid sequences and nucleic acid sequences do not mutate at the same rate. Due to the degenerate nature of DNA, bases can change without affecting the amino acid sequence. For example, there are six codons that code for leucine. Thus, despite the difference in mutation rates, it is essential to incorporate nucleic acid evolution into the discussion of protein evolution. At the end of the 1960s, two groups of scientists—Kimura (1968) and King and Jukes (1969)—independently proposed that a majority of the evolutionary changes observed in proteins were neutral. Since then, the neutral theory has been expanded upon and debated.

B. Nucleotide Evolution:

Molecular evolution is the process of change in the sequence composition of cellular molecules such as DNA, RNA, and proteins across generations. The field of molecular evolution uses principles of evolutionary biology and population genetics to explain patterns in these changes. Major topics in molecular evolution concern the rates and impacts of single nucleotide changes, neutral evolution vs. natural selection, origins of new genes, the genetic nature of complex traits, the genetic basis of speciation, evolution of development, and ways that evolutionary forces influence genomic and phenotypic changes.

The history of molecular evolution starts in the early 20th century with comparative biochemistry, and the use of "fingerprinting" methods such as immune assays, gel electrophoresis and paper chromatography in the 1950s to explore homologous proteins. The field of molecular evolution came into its own in the 1960s and 1970s, following the rise of molecular biology. The advent of protein sequencing allowed molecular biologists to create phylogenies based on sequence comparison, and to use the differences between homologous sequences as a molecular clock to estimate the time since the last universal common ancestor.

In the late 1960s, the neutral theory of molecular evolution provided a theoretical basis for the molecular clock, though both the clock and the neutral theory were controversial, since most evolutionary biologists held strongly to panselectionism, with natural selection as the only important cause of evolutionary change. After the 1970s, nucleic acid sequencing allowed molecular evolution to reach beyond proteins to highly conserved ribosomal RNA sequences, the foundation of a reconceptualization of the early history of life.

Forces in molecular evolution:

The content and structure of a genome is the product of the molecular and population genetic forces which act upon that genome. Novel genetic variants will arise through mutation and will spread and be maintained in populations due to genetic drift or natural selection.

a. Mutation: Mutations are permanent, transmissible changes to the genetic material (DNA or RNA) of a cell or virus. Mutations result from errors in DNA replication during cell division and by exposure to radiation, chemicals, and other environmental stressors, or viruses and transposable elements. Most mutations that occur are single nucleotide polymorphisms which modify single bases of the DNA sequence, resulting in point mutations. Other types of mutations modify larger segments of DNA and can cause duplications, insertions, deletions, inversions, and translocations. Most organisms display a strong bias in the types of mutations that occur with strong influence in GC-content. Transitions (A ↔ G or C ↔ T) are more common than transversions (purine (adenine or guanine)) ↔ pyrimidine (cytosine or thymine, or in RNA, uracil) and are less likely to alter amino acid sequences of proteins.

Mutations are stochastic and typically occur randomly across genes. Mutation rates for single nucleotide sites for most organisms are very low, roughly 10^{-9} to 10^{-8} per site per generation, though some viruses have higher mutation rates on the order of 10^{-6} per site per generation. Among these mutations, some will be neutral or beneficial and will remain in the genome unless lost via genetic drift, and others will be detrimental and will be eliminated from the genome by natural selection. Because mutations are extremely rare, they accumulate very slowly across generations. While the number of mutations which appears in any single generation may vary, over very long time periods they will appear to accumulate at a regular pace. Using the mutation rate per generation and the number of nucleotide differences between two sequences, divergence times can be estimated effectively via the molecular clock.

b. Recombination: Recombination is a process that results in genetic exchange between chromosomes or chromosomal regions. Recombination counteracts physical linkage between adjacent genes, thereby reducing genetic hitchhiking. The resulting independent inheritance of genes results in more efficient selection, meaning that regions with higher recombination will harbour fewer detrimental mutations, more selectively favoured variants, and fewer errors in replication and repair. Recombination can also generate particular types of mutations if chromosomes are misaligned.

c. Gene conversion: Gene conversion is a type of recombination that is the product of DNA repair where nucleotide damage is corrected using an homologous genomic region as a template. Damaged bases are first excised, the damaged strand is then aligned with an undamaged homolog, and DNA synthesis repairs the excised region using the undamaged strand as a guide. Gene conversion is often responsible for homogenizing sequences of duplicate genes over long time periods, reducing nucleotide divergence.

d. Genetic drift: Genetic drift is the change of allele frequencies from one generation to the next due to stochastic effects of random sampling in finite populations. Some existing variants have no effect on fitness and may increase or decrease in frequency simply due to chance. "Nearly neutral" variants whose selection coefficient is close to a threshold value of 1 / the effective population size will also be affected by chance as well as by selection and mutation. Many genomic features have been ascribed to accumulation of nearly neutral detrimental mutations as a result of small effective population sizes. With a smaller effective population size, a larger variety of mutations will behave as if they are neutral due to inefficiency of selection.

e. Selection: Selection occurs when organisms with greater fitness, i.e. greater ability to survive or reproduce, are favoured in subsequent generations, thereby increasing the instance of underlying genetic variants in a population. Selection can be the product of natural selection, artificial selection, or sexual selection. Natural selection is any selective process that occurs due to the fitness of an organism to its environment. In contrast sexual selection is a product of mate choice and can favour the spread of genetic variants which act counter to natural selection but increase desirability to the opposite sex or increase mating success. Artificial selection, also known as selective breeding, is imposed by an outside entity, typically humans, in order to increase the frequency of desired traits. The principles of population genetics apply similarly to all types of selection, though in fact each may produce distinct effects due to clustering of genes with different functions in different parts of the genome, or due to different properties of genes in particular functional classes. For instance, sexual selection could be more likely to affect molecular evolution of the sex chromosomes due to clustering of sex specific genes on the X, Y, Z or W.

Intragenomic conflict: Selection can operate at the gene level at the expense of organismal fitness, resulting in intragenomic conflict. This is because there can be a selective advantage for selfish genetic elements in spite of a host cost. Examples of such selfish elements include transposable elements, meiotic drivers, killer X chromosomes, selfish mitochondria, and self-propagating introns.

Genome architecture:

Genome size: Genome size is influenced by the amount of repetitive DNA as well as number of genes in an organism. The C-value paradox refers to the lack of correlation between organism 'complexity' and genome size. Explanations for the so-called paradox are two-fold. First, repetitive genetic elements can comprise large portions of the genome for many

organisms, thereby inflating DNA content of the haploid genome. Secondly, the number of genes is not necessarily indicative of the number of developmental stages or tissue types in an organism. An organism with few developmental stages or tissue types may have large numbers of genes that influence non-developmental phenotypes, inflating gene content relative to developmental gene families. Neutral explanations for genome size suggest that when population sizes are small, many mutations become nearly neutral. Hence, in small populations repetitive content and other 'junk' DNA can accumulate without placing the organism at a competitive disadvantage. There is little evidence to suggest that genome size is under strong widespread selection in multicellular eukaryotes. Genome size, independent of gene content, correlates poorly with most physiological traits and many eukaryotes, including mammals, harbour very large amounts of repetitive DNA.
However, birds likely have experienced strong selection for reduced genome size, in response to changing energetic needs for flight. Birds, unlike humans, produce nucleated red blood cells, and larger nuclei lead to lower levels of oxygen transport. Bird metabolism is far higher than that of mammals, due largely to flight, and oxygen needs are high. Hence, most birds have small, compact genomes with few repetitive elements. Indirect evidence suggests that non-avian theropod dinosaur ancestors of modern birds also had reduced genome sizes, consistent with endothermy and high energetic needs for running speed. Many bacteria have also experienced selection for small genome size, as time of replication and energy consumption are so tightly correlated with fitness.

Repetitive elements : Transposable elements are self-replicating, selfish genetic elements which are capable of proliferating within host genomes. Many transposable elements are related to viruses, and share several proteins in common.

Chromosome number and organization: The number of chromosomes in an organism's genome also does not necessarily correlate with the amount of DNA in its genome. The ant *Myrmecia pilosula* has only a single pair of chromosomes whereas the Adders-tongue fern *Ophioglossum reticulatum* has up to 1260 chromosomes. Cilliate genomes house each gene in individual chromosomes, resulting in a genome which is not physically linked. Reduced linkage through creation of additional chromosomes should effectively increase the efficiency of selection. Changes in chromosome number can play a key role in speciation, as differing chromosome numbers can serve as a barrier to reproduction in hybrids. Human chromosome 2 was created from a fusion of two chimpanzee chromosomes and still contains central telomeres as well as a vestigial second centromere. Polyploidy, especially allopolyploidy, which occurs often in plants, can also result in reproductive incompatibilities with parental species. Agrodiatus blue butterflies have diverse chromosome numbers ranging from n=10 to n=134 and additionally have one of the highest rates of speciation identified to date.

Gene content and distribution : Different organisms house different numbers of genes within their genomes as well as different patterns in the distribution of genes throughout the genome. Some organisms, such as most bacteria, Drosophila, and Arabidopsis have particularly compact genomes with little repetitive content or non-coding DNA. Other organisms, like mammals or maize, have large amounts of repetitive DNA, long introns, and substantial spacing between

different genes. The content and distribution of genes within the genome can influence the rate at which certain types of mutations occur and can influence the subsequent evolution of different species. Genes with longer introns are more likely to recombine due to increased physical distance over the coding sequence. As such, long introns may facilitate ectopic recombination, and result in higher rates of new gene formation.

Origins of new genes: New genes arise from several different genetic mechanisms including gene duplication, de novo origination, retrotransposition, chimeric gene formation, recruitment of non-coding sequence, and gene truncation. Gene duplication initially leads to redundancy. However, duplicated gene sequences can mutate to develop new functions or specialize so that the new gene performs a subset of the original ancestral functions. In addition to duplicating whole genes, sometimes only a domain or part of a protein is duplicated so that the resulting gene is an elongated version of the parental gene. Retrotransposition creates new genes by copying mRNA to DNA and inserting it into the genome. Retrogenes often insert into new genomic locations, and often develop new expression patterns and functions. Chimeric genes form when duplication, deletion, or incomplete retrotransposition combine portions of two different coding sequences to produce a novel gene sequence. Chimeras often cause regulatory changes and can shuffle protein domains to produce novel adaptive functions.

De novo gene birth can also give rise to new genes from previously non-coding DNA. For instance, Levine and colleagues reported the origin of five new genes in the D. melanogaster genome from noncoding DNA. Similar de novo origin of genes has been also shown in other organisms such as yeast, rice and humans. De novo genes may evolve from transcripts that are already expressed at low levels. Mutation of a stop codon to a regular codon or a frameshift may cause an extended protein that includes a previously non-coding sequence. The formation of novel genes from scratch typically cannot occur within genomic regions of high gene density. The essential events for de novo formation of genes is recombination/mutation which includes insertions, deletions, and inversions. These events are tolerated if the consequence of these genetic events does not interfere in cellular activities. Most genomes comprise prophages wherein genetic modifications do not, in general, affect the host genome propagation. Hence, there is higher probability of genetic modifications, in regions such as prophages, which is proportional to the probability of de novo formation of genes. De novo evolution of genes can also be simulated in the laboratory. For example, semi-random gene sequences can be selected for specific functions. More specifically, they selected sequences from a library that could complement a gene deletion in E. coli. The deleted gene encodes ferric enterobactin esterase (Fes), which releases iron from an iron chelator, enterobactin. While Fes is a 400 amino acid protein, the newly selected gene was only 100 amino acids in length and unrelated in sequence to Fes.

The driving forces of evolution:

Depending on the relative importance assigned to the various forces of evolution, three perspectives provide evolutionary explanations for molecular evolution.

Selectionist hypotheses argue that selection is the driving force of molecular evolution. While acknowledging that many mutations are neutral, selectionists attribute changes in the frequencies of neutral alleles to linkage disequilibrium with other loci that are under selection, rather than to random genetic drift. Biases in codon usage are usually explained with reference to the ability of even weak selection to shape molecular evolution.

Neutralist **hypotheses** emphasize the importance of mutation, purifying selection, and random genetic drift. The introduction of the neutral theory by Kimura, quickly followed by King and Jukes' own findings, led to a fierce debate about the relevance of neodarwinism at the molecular level. The Neutral theory of molecular evolution proposes that most mutations in DNA are at locations not important to function or fitness. These neutral changes drift towards fixation within a population. Positive changes will be very rare, and so will not greatly contribute to DNA polymorphisms. Deleterious mutations do not contribute much to DNA diversity because they negatively affect fitness and so are removed from the gene pool before long. This theory provides a framework for the molecular clock. The fate of neutral mutations are governed by genetic drift, and contribute to both nucleotide polymorphism and fixed differences between species. In the strictest sense, the neutral theory is not accurate. Subtle changes in DNA very often have effects, but sometimes these effects are too small for natural selection to act on. Even synonymous mutations are not necessarily neutral because there is not a uniform amount of each codon. The nearly neutral theory expanded the neutralist perspective, suggesting that several mutations are nearly neutral, which means both random drift and natural selection is relevant to their dynamics. The main difference between the neutral theory and nearly neutral theory is that the latter focuses on weak selection, not strictly neutral.

Mutationists hypotheses emphasize random drift and biases in mutation patterns. Sueoka was the first to propose a modern mutationist view. He proposed that the variation in GC content was not the result of positive selection, but a consequence of the GC mutational pressure.

Molecular Clocks : The key to using biological molecules as molecular clocks is the hypothesis of neutral evolution. This hypothesis states that most of the variability in molecular structure does not affect the molecule's functionality. This is because most of the variability occurs outside of the functional regions of the molecule. Changes that do not affect functionality are called "neutral substitutions" and their accumulation is not affected by natural selection. As a result, neutral substitutions occur at a fairly regular rate, though that rate is different for different molecules. Not every molecule makes a good molecular clock, however. To serve as a molecular clock, a molecule must meet two requirements: 1) it must be present in all of the organisms being studied; 2) it must be under strong functional constraint so that the functional regions are highly conserved. Examples of molecules that have been used to study evolution are cytochrome c, which is vital to the respiratory pathway, and ribosomal RNA, which performs protein synthesis.

Once a good molecular clock is identified, using it to compare species is fairly simple. The most complicated step is the comparison of molecular sequences. The sequences of the molecule in the different species must be compared so that the number of amino acid or nucleic

acid bases that differ can be counted. This number is then plotted against the rate at which the molecule is known to undergo neutral base pair substitutions to determine the point at which two species last shared a common ancestor. Depending on the rate of substitution, molecules may be used to determine ancient relationships or relatively recent ones. Ribosomal RNA has a very slow rate of substitution, so it is most commonly used in conjunction with fossil information to determine relationships between extremely ancient species.

Probable questions:

1. How proteins have evolved? State briefly with example.
2. What s the relation between protein and nucleotide evolution?
3. What are the forces which accelerated molecular evolution?
4. How new genes have originated?
5. Define and explain molecular clock.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

Unit-VIII

Gene frequencies and equilibrium: gene frequencies; gene frequencies, gene pool, conservation of gene frequencies

Objective: In this unit we will discuss about gene pool, gene and genotype frequency and also about conservation of gene frequencies.

Gene Pool: The gene pool are all the different alleles that are present in a population. For example; you have a population of bunnies of different colours. The colours are caused by different variations of the same gene. All the different variations of the gene make up the gene pool. The gene pool does not bother with frequencies; a variant is either present or not. You can compare the size of the gene pool between populations or in the course of time. When there are more gene variants present in a population, the gene pool is big. It can get smaller because of stochasticity (e.g. a bottleneck in population size accidentally losing all the individuals with a certain allele), selective breeding or natural selection. It can get bigger because of mutations or introduction of individuals of the same species from a different population.

Measurement of Gene pool : If you want to measure the gene pool, you need to know how many variant of a gene are present in a given population. To do so you need to sample the DNA of a certain amount of individuals in a population. Then you can use DNA sequencing or gel electrophoresis to determine how many variants of the gene are present. You can really only look at one gene or a set of genes at one time. Usually scientists look at genes that are not selected for (neutral genes) or genes that are very important for survival chances of the population (like variation in immune defence genes). The size of the gene pool is a direct measure of the amount of genetic variation.

What happens if the gene pool gets smaller?

If the gene pool of a population gets smaller you are stating that the amount of genetic variation of a population goes down. In itself this is not bad and it is happening all the time, as natural selection filters out some genetic variation. The problem comes from a too small gene pool. Low genetic variation causes the population to be vulnerable to changes in the environment and stochasticity. When a population has a low variation, there are fewer genes to select on if the environment changes. E.g. if you have a population of only black rabbits they will be more vulnerable to predation when there is snow. A bigger gene pool, with black, white and grey rabbits, gives natural selection a hand hold to select on.

Population Genetics:

A population consisting of sexually interbreeding organisms carrying one or more particular genes, which follow the Mendelian Principles of Inheritance, is called ‘Mendelian Population’. Gene pool and gene frequencies are considered to be two important attributes of a population.

A gene pool is the sum total of genes in reproductive gametes of a population. The nature of gene pool depends on random mating of gametes to form zygotes in the next generation.
Gene frequency can be defined as proportions of different alleles of a gene in a population, and in a particular generation these frequencies depend on their frequencies in the previous generation and also on the proportion of various genotypes in total population.
In any population, if a character is controlled by two alleles, then the frequency of these alleles or genes can be calculated very easily by phenotypic observation of that character under homozygous and heterozygous conditions. The frequency of an allele in a population is the number of occurrence of that allele divided by the total number of alleles of that gene locus.
Regarding the genetic structure of the population, the following two hypotheses have been proposed:- **1. Classical Hypothesis 2. Balance Hypothesis.**

a. Classical Hypothesis:
It was developed by T.H. Morgan (1932) and supported by H.J. Muller and Kaplan (1966). The classical hypothesis proposes that the gene pool of a population consists at each gene locus of a wild-type allele with a frequency approaching one.
Mutant alleles in very low frequencies may also exist at each locus. A typical individual would be homozygous for the wild-type allele at most gene loci; at a very small proportion of its loci, the individual would be heterozygous for a wild and a mutant allele.
Except in the progenies of consanguineous mating, individuals homozygous for a mutant allele would be extremely rare. The "normal" ideal genotype would be an individual homozygous for the wild-type allele.
According to classical hypothesis, mutant alleles are continuously introduced in the population by mutation pressure, but are generally deleterious and, thus, are more or less gradually removed from the population by natural selection. Periodically, a beneficial mutant allele might arise, conferring higher fitness upon its carriers than the pre-existing wild-type allele.
This beneficial allele would gradually increase in frequency by natural selection to become the new wild-type allele, while the former wild-type allele would be eliminated. Evolution, thus, consists of the replacement at an occasional locus of the pre-existing wild-type allele by a new wild-type allele.

Balance Hypothesis:
This hypothesis was proposed by Dobzhansky (1970) and E.B. Ford (1971). This hypothesis was derived by direct study of natural populations. According to the balance model, there is generally no single wild-type or 'normal' allele. Rather, the gene pool of a population is envisioned as consisting at most loci of an array of alleles in moderate frequencies.
A typical individual is heterozygous at a large proportion of its gene loci. There is no 'normal' or ideal genotype, only an adaptive norm consisting of an array of genotypes that yield a satisfactory fitness in most environments encountered by the population.
The proponents of the balance hypothesis argue that the common allelic polymorphisms are maintained in populations by various forms of balancing natural selection. The fitness granted on its carriers by an allele depends on what other alleles exist in the genotype at that and other gene loci. It also depends, of course, on the environment.

Gene pools are co-adapted systems; the sets of alleles favoured at one locus depend on the sets of alleles that exist at other loci. Evolution occurs by gradual change in the frequencies and kinds of alleles at many gene loci. As the configuration of the set of alleles changes at one locus, it also changes at many other loci.
The balance model of genetic structure of populations has now become definitely established, although some controversy remains regarding the process maintaining the common polymorphisms.

Hardy Weinberg's Law:

In 1908, the mathematician G. H. Hardy in England and the physician W. Weinberg in Germany independently developed a quantitative theory for defining the genetic structure of populations. The Hardy-Weinberg Law provides a basic algebraic formula for describing the expected frequencies of various genotypes in a population.
The similarity of their work however, remained unnoticed until Stern (1943) drew attention to both papers and recommended that names of both discoverers be attached to the population formula. The Law states that gene frequencies in a population remain constant from generation to generation if no evolutionary processes like migration, mutation, selection and drift are operating.Thus if matings are random, and no other factors disturb the reproductive abilities of any genotype, the equilibrium genotypic frequencies are given by the square of the allelic frequencies.

If there are only two alleles A and a with frequencies p and q respectively, the frequencies of the three possible genotypes are:

$$(p + q)^2 = p^2 + 2pq + q^2$$

If there are 3 alleles say A1, A2 and A3 with frequencies p, q and r, the genotypic frequencies would be; $(p + q + r)^2 = p^2 + q^2 + r^2 + 2pq + 2pr + 2qr$

This square expansion can be used to obtain the equilibrium genotypic frequencies for any number of alleles. It must also be noted that the sum of all the allelic frequencies, and of all the genotypic frequencies must always be 1. If there are only two alleles p and q, then $p + q = 1$, and therefore $p^2 + 2pq + q^2 = (p + q)^2 = 1$. If there are 3 alleles with frequencies p, q, and r, then $p + q + r = 1$, as well as $(p + q + r)^2 = 1$.

The time required for attaining equilibrium frequencies has been determined. If a certain population of individuals with one set of allele frequencies mixes with another set and complete panmixis occurs (that is, random mating), then the genotypes of the next generation will be found in the proportion $p^2 + 2pq + q^2$ where p and q are allele frequencies in the new mixed populations. Thus it takes only one generation to reach Hardy-Weinberg equilibrium provided the allelic frequencies are the same in males and females. If the allelic frequencies are different in the two sexes, then they will become the same in one generation in the case of alleles on autosomes, and genotypic frequencies will reach equilibrium in two generations.

In general equilibrium is arrived at within one or at the most a few generations. Once equilibrium is attained it will be repeated in each subsequent generation with the same frequencies of alleles and of genotypes.
The Hardy-Weinberg law is applicable when there is random mating. Random mating occurs in a population when the probability of mating between individuals is independent of their genetic constitution. Such a population is said to be panmictic or to undergo panmixis. The matings between the genotypes occur according to the proportions in which the genotypes are present.
The probability of a given type of mating can be found out by multiplying the frequencies of the two genotypes that are involved in the mating. Mating are not random for instance when a population consists of different races such as blacks and whites in the U.S., or different communities as in India as there are preferred mating between members of the same racial or communal group.

Assumptions of Hardy-Weinberg Equilibrium:
We will consider a population of diploid, sexually reproducing organisms with a single autosomal locus segregating two alleles (i.e., every individual is one of three genotypes – MM, MN and AW).

The following major assumptions are necessary for the Hardy-Weinberg equilibrium to hold:
1. Random Mating,
2. Large Population Size,
3. No Mutation or Migration, and
4. No Natural Selection.

1. Random Mating:
The first assumption of Hardy-Weinberg equilibrium is random mating which means that the probability that two genotypes will mate is the product of the frequencies (or probabilities) of the genotypes in the population.
If an MM genotypes makes up 90% of a population, then any individual has a 90% chance (probability = 0.9) of mating with a person with an MM genotype. The probability of an MM by MM mating is (0.9) (0.9), or 0.81.
Any deviation from random mating comes about for two reasons: choice or circumstance. If members of a population choose individuals of a particular phenotype as mates more or less often than at random, the population is engaged in assortative mating.
If individuals with similar phenotypes are mating more often than at random, positive assortative mating is in force; if mating occur between individuals with dissimilar phenotypes more often than at random, negative assortative mating or disassortative mating is at work.
Further, deviation from random mating also arise when mating individuals are either more closely related genetically or more distantly related than individuals chosen at random from the population.

Inbreeding is the mating of related individuals, and outbreeding is the mating of genetically unrelated individuals. Inbreeding is a consequence of pedigree relatedness (e.g., cousins) and small population size.

One of the first distinct observations of population genetics is that deviation from random mating alter genotypic frequencies but not allelic frequencies. Imagine a population in which every individual is the parent of two children on the average, each individual will pass on one copy of each of his or her alleles.

Assortative mating and inbreeding will change the zygotic (genotypic) combinations from one generation to the next, but will not change which alleles are passed into the next generation. Thus, genotypic, but not allelic frequencies change under non-random mating.

2. Large Population Size:

Although an extremely large number of gametes are produced in each generation, each successive generation is the result of a sampling of a relatively small portion of the gametes of the previous generation. A sample may not be an accurate representation of a population, especially if the sample is small.

Thus, the second assumption of the Hardy-Weinberg equilibrium is that the population is infinitely large. A large population produces a large sample of successful gametes. The larger the sample of successful gametes, the greater the probability that the allelic frequencies of the offspring will accurately represent the allelic frequencies in the parental population.

When populations are small or when alleles are rare, changes in allelic frequencies take place due to chance alone. These changes are referred to as random genetic drift or just genetic drift.

3. No Mutation or Migration:

Allelic and genotypic frequencies may change through the loss or addition of alleles through mutation or migration (immigration or emigration) of individuals from or into a population, ne third and fourth assumptions of the Hardy-Weinberg equilibrium are that neither mutation nor migration causes such allelic loss or addition in the population.

4. No Natural Selection:

The final assumption necessary to the Hardy-Weinberg equilibrium is that no individual will have a reproductive advantage over another individual because of its genotype. In other words, no natural selection in occurring. (Note. Artificial selection, as practised by animal and plant breeders, will also perturb the Hardy-Weinberg equilibrium of captive population).

The significance of Hardy-Weinberg equilibrium was not immediately appreciated. A rebirth of biometrical genetics was later brought about with the classical papers of R.A. Fisher, beginning in 1918 and those of Sewall Wright, beginning in 1920.

Under the leadership of these mathematicians, emphasis was placed on the population rather than on the individual or family group, which had previously occupied the attention of most Mendelian geneticists. In about 1935, T. Dobzhansky and others started to interpret and to popularize the mathematical approach for studies of genetics and evolution.

Genetic Equilibrium:

As shown by Hardy and Weinberg, alleles segregating in a population tend to establish an equilibrium with reference to each other. Thus, if two alleles should occur in equal proportion in a large, isolated breeding population and neither had a selective or mutational advantage over the other, they would be expected to remain in equal proportion generation after generation. This would be a special case because alleles in natural populations seldom if ever, occur in equal frequency. They may, however, be expected to maintain their relative frequency, whatever it is, subject only to such factors as chance, natural selection, differential mutation rates or mutation pressure, meiotic drive and migration pressure, all of which alter the level of the allele frequencies. A genetic equilibrium is maintained through random mating.

Applications of the Hardy-Weinberg Law:

(a) Complete Dominance:

When Hardy-Weinberg equilibrium exists, allele frequencies can even be found out in presence of complete dominance where two genotypes cannot be distinguished. If two genotypes AA and Aa have the same phenotype due to complete dominance of A over a the allele frequencies can be determined from the frequencies of individuals showing the recessive phenotype aa.

The frequency of aa individuals must be equal to the square of the frequency of the recessive allele q. Let us suppose q = 0.5, then $q^2 – (0.5)^2 = 0.25$. In other words when aa phenotype is 0.25 in the population, then it follows that the frequency of the recessive allele a is $\sqrt{0.25} – 0.5$. The frequency of the dominant allele A would be 1 – q or 1 – 0.25 = 0.75.

(b) Frequencies of Harmful Recessive Alleles:

The Hardy-Weinberg Law can also be used to calculate the frequency of heterozygous carriers of harmful recessive genes. If there are two alleles A and a at an autosomal locus with frequencies p and q in the population and p + q = 1, then the frequency of AA, Aa, and aa genotypes would be $p^2 + 2pq + p^2$.

If the aa genotype expresses a harmful phenotype such as cystic fibrosis, then the proportion of affected individuals in the population would be q^2, and the frequency of the heterozygous carriers of the recessive allele would be 2pq.

To illustrate with figures, suppose one out of 1,000 children is affected with cystic fibrosis, then the frequency $q^2 = 0.001$, so that $q = \sqrt{0.001}$ which is about 0.032, then 2pq = 2 x 0.032 x 0.968 = 0.062. This means that about 62 individuals out of 1000 or one out of 16 is a carrier of the allele for cystic fibrosis. As already mentioned the number of individuals (aa) who are actually affected is one out of 1000. This implies that the frequency of heterozygous carriers is much higher than that of affected homozygotes. Similar calculation shows that when an allele is very rare in the population the proportion of carriers is still much higher and of affected homozygotes much lower. Thus, lower the frequency of an allele, greater the proportion of that allele that exists in the heterozygotes.

(c) Multiple Alleles:

The Hardy-Weinberg Law permits calculation of genotypic frequencies at loci with more than two alleles, such as the ABO blood groups. There are 3 alleles I^A, I^B and I° with frequencies p,

q and r. Here p + q + r = 1. The genotypes of a population with random mating would be $(p + q + r)^2$.

(d) Sex-Linked Loci:

It is possible to apply Hardy-Weinberg Law for calculating gene frequencies in case of sex-linked loci in males and females. Red green colour blindness is a sex- linked recessive trait. Let r denote the recessive allele which produces affected individuals, and R the normal allele. The frequency of R is p and of r is q where p + q = 1. The frequencies of females having RR, Rr, rr genotypes would be p^2, 2pq, q^2 respectively.

Males are different as they are hemizygous, have only one X chromosome derived from the mother with a single allele either R or r. The frequency of affected r males would be the same as the frequency of the r allele among the eggs that is q. The frequency of normal R males would be p. Suppose the frequency of r alleles is 0.08, then the incidence of affected males would be 0.08 or about 8%. The frequency of affected rr females would be $(0.08)^2 = 0.0064$ or 0.64%. Thus the Hardy-Weinberg Law explains that males would be affected a hundred times more frequently than females. This is actually what is observed. Males are more affected by sex-linked recessive traits than females. The difference between the sexes is even more pronounced if the recessive allele is still more rare. The incidence of a common form of haemophilia is one in a thousand males; thus q = 0.001. However, only one in 1000,000 females will be affected. Thus males could have haemophilia one thousand times more often than females.

(e) Linkage Disequilibrium:

Consider two or more alleles at one locus and another locus on the same chromosome with two or more alleles. Due to genetic exchange by recombination occurring regularly over a period of time, the frequencies of the allelic combinations at the two syntenic loci will reach equilibrium. If equilibrium is not reached, the alleles are said to be in linkage disequilibrium. The effect is due to tendency of two or more linked alleles to be inherited together more often than expected. Such groups of genes have also been referred to as supergenes.

Measurement of Gene Frequency:

In a diploid species, a population having N individuals has 2N alleles for each gene locus. If there are two alleles ‘A’ and ‘a’ of a particular gene in this population, the number of A alleles is twice the number of AA homozygotes plus the number of Aa heterozygotes, as each homozygote has two ‘A’ alleles, and each heterozygote has one ‘A’ allele. So the frequency of ‘A’ is the number of ‘A’ alleles divided by total number of alleles, i.e., 2N.

If the number is denoted by 'n' then the equation can be written as:

$$n_A = 2n_{AA} + n_{Aa}$$

$$n_a = 2n_{aa} + n_{Aa}$$

If the **frequency of allele A** is denoted by 'p' then.

$$p = \frac{n_A}{2N} = \frac{2n_{AA} + n_{Aa}}{2N}$$

Similarly, if the **frequency of allele a** is denoted by 'q' then.

$$q = \frac{n_a}{2N} = \frac{2n_{aa} + n_{Aa}}{2N}$$

It must be remembered that for all alleles the total frequency will be always 1, i.e., $p + q = 1$ and $n_A + n_a = 2N$.

Example 1:

In human population, a sample of 100 individuals for MN blood group character shows 50MM, 20MN and 30 NN individuals, then the frequency of M and N allele can be calculated using the above formula.

The frequency of 'M' will be $= \frac{2 \times 50 + 20}{200} = \frac{120}{200} = 0.6$

where $2n_{MM} = 2 \times 50$ $n_{MN} = 20.$ $2N = 200.$

The frequency of 'N' will be $= \frac{2 \times 30 + 20}{200} = \frac{80}{200} = 0.4$

where $2n_{NN} = 2 \times 30.$ $n_{MN} = 20.$ $2N = 200.$

The frequency can be calculated by another formula:

Frequency of a gene = frequency of homozygotes of that gene

$+ \frac{1}{2}$ frequency of heterozygotes

In the above example,

Frequency of M = 0.5 MM + $\frac{1}{2}$ (0.2 MN) = 0.6

Frequency of N = 0.3 NN + $\frac{1}{2}$ (0.2 MN) = 0.4.

Genotype Frequency and Hardy-Weinberg Equilibrium:

In a randomly mating Mendelian population the gene and genotype frequencies reach to equilibrium in a single generation. The Hardy-Weinberg law states that the gene and genotype frequencies in a Mendelian population remain constant generation after generation if there is no selection, mutation, migration or genetic drift.

Homozygotes (AA & aa) are produced by the union of gametes carrying similar alleles. The frequency with which a male gamete carrying allele A (frequency p) fuses with a female gamete carrying 'A' (also frequency p) will be $p \times p = p^2$. Similarly the frequency with which a male gamete carrying allele 'a' (frequency q)"fuses with a female gamete also carrying 'a' will be $q \times q = q^2$. Heterozygotes (Aa) are produced by the fusion of gametes carrying different alleles, the frequency with which a male gamete carrying allele 'A' fuses with a female gamete 'a' will be $p \times q = pq$. Similarly in opposite way the frequency with which a female gamete carrying

allele ‘A’ fuses with a male gamete carrying ‘a’ will be p x q = pq. So, the total frequency of heterozygotes will be 2 pq.

♂ \ ♀	A (p)	a (q)
A(p)	AA (p^2)	Aa (pq)
a (q)	Aa (pq)	aa (q^2)

Fig. 14.1: The frequencies of three genotypes produced due to random mating in a population having ‘A’ allele with frequency ‘p’ and allele ‘a’ with frequency ‘q’

According to the theory of probability, due to random mating, the genotype frequency can easily be calculated from the following formula:

$(p + q) \times (p + q) = p^2 + 2pq + q^2$

(p + q) = Total frequency of two alleles ‘A’ and ‘a’.

This equation is called Hardy-Weinberg equation which is named after British Mathematician G.H. Hardy and German Physician W. Weinberg.

As according to theory of probability the total frequency of p + q is always 1. So,

$(p + q)^2 = p^2 + 2pq + q^2 = 1$

In case of genes showing co-dominance, this equation can be used very easily to calculate the gene frequency by observing the phenotypic characters of homozygotes and heterozygotes.

Example 2:

In the following example it is shown how the observed value help to calculate the frequency:

MN blood group of Human

Phenotype	Genotype	No. of Individuals	Frequency Calculation
M	L_ML_M	1787	$L_M = \frac{2 \times 1787 + 3039}{2 \times 6129} = 0.5395$
MN	L_ML_N	3039	
N	L_NL_N	1303	$L_N = \frac{2 \times 1303 + 3039}{2 \times 6129} = 0.4605$
	Total	6129	

If we put these two values in a frequency table we can get the expected number of phenotypes due to random mating of each allele as follows:

	L_M p = 0.5395	L_N q = 0.4605
L_M p = 0.5395	p^2 = 0.2911	pq = 0.2484
L_N q = 0.4605	pq = 0.2484	q^2 = 0.2121

So, the expected values in total population can be calculated

$$p^2 = 0.2911 \times 6129 = 1784.2$$
$$2pq = 2 \times 0.2484 \times 6129 = 3044.8$$

$$q^2 = 0.2121 \times 6129 = 1300$$

The above calculation shows that the observed number of individuals and expected number of phenotypes are very close to each other which follows the Hardy-Weinberg Equilibrium Frequencies. In case of completely dominant genes of a population, the heterozygotes cannot be phenotypically distinguished from homozygotes for the dominant character.

In this case, by observing the recessive phenotype, the recessive allele frequency can be measured very easily using Hardy-Weinberg equation. The frequency of homozygotes of recessive allele is q^2. So the frequency of recessive allele in a population is $\sqrt{q^2} = q$.

The frequency of dominant allele in that population is $(1 – q) = p$. This concept can also be applied to determine the number of individuals affected or carrying a particular disease, vis-a-vis, the frequency of the gene causing the disease.

PKU (Phenylketonuria) is a serious disease of human being which is a genetically controlled metabolic disorder. If in any population 0.04% is PKU affected then the frequency of PKU allele in that population is 7.0004 = 0.02 and the frequency of dominant allele is (1 – 0.02) = 0.98. If in a population the frequency of deleterious allele is 0.01, assuming random mating the frequency of homozygous population will be $(0.01)^2 = .0001$. Now the heterozygous frequency will be 2 x .01 x (1 – 0.01) = 2 x .01 x 0.99 = 0.0198.

So, about 1.98% population are carrier of this allele in heterozygous condition which is not expressed. If the frequency of recessive allele is too small then it is very difficult to measure it correctly, thus it is desirable to draw a large sample which will give more error free estimation.

Forces that Change Gene Frequency in Population. Four major forces are usually listed for changing gene frequencies in populations, namely migration, mutation, selection and random genetic drift. These forces constitute the mechanisms underlying the evolutionary process.

1. Migration:

Migration occurs when a large influx of people moves into another population and interbreeds with the latter. The phenomenon called gene flow takes place if one population contributes an allele to the other population. Let us suppose that a migrating population m interbreeds with members of another population.

Then the descendants of the next generation will have m genes from the migrants and 1 – m genes from members of the original population. Consider an allele A occurring with frequency p in migrant population.

In the original population this allele has frequency q. In the next generation the frequency of A in the new population would be:

$$r = (1 – m)q + mp$$

$$= q – m (q – p)$$

That means the frequency of allele A in the new population now would be the original allelic frequency q multiplied by the genes (1 – m) present in the original population plus the product of reproducing migrant individuals and their gene frequency imp). Thus there will be a new gene frequency in the next generation.

Migration is a complex phenomenon in humans, influenced by many factors. It seems however, that it leads to make populations genetically more similar than they would be otherwise.

2. Mutation:

The ultimate source of all genetic variation is mutation. Both chromosomal rearrangements and point mutations are implied here as they follow the same rules of population dynamics. However, mutations occur with an extremely low frequency. In humans where there may be from 30-50 successive mitotic divisions in the germ cells in each generation, only one gene in a million or 10 million roughly undergoes a mutation.

Consider an allele A that is homozygous in many individuals in a population. Assume that in every generation one A allele in a million mutates to a. This will reduce the frequency of A allele over many generations, while a allele will gradually accumulate in the population. The change in frequencies of A and a occurs at an unimaginably slow rate.

The a allele however, can also back mutate to A; this event will take place as the frequency of a alleles increases. After a very long time the number of A alleles lost by forward mutation would be balanced by the number of A alleles arising from back mutation of a to A. When this happens gene frequencies of A and a are said to be in mutation equilibrium. Thereafter, no further change in

can be affected by environmental factors like radiation and chemicals. In some instances mutations are under genetic control. There is a recessive mutator gene on the second chromosome of Drosophila melanogaster. In stocks of flies homozygous for the mutator gene, sex-linked recessive lethals occur spontaneously with high frequency. In maize the recessive mutator gene frequency of A and a will occur in subsequent generations. This applies however, only when the other evolutionary forces such as migration, selection and genetic drift are not operating to affect gene frequencies in the population.

Experimental work has shown that the rate of mutation Dt acts on an unlinked locus a which controls synthesis of the purple anthocyanin pigment. The mutator gene Dt causes mutation of recessive a alleles to the dominant form A which leads to synthesis of anthocyanin appearing as purple spots on the stem, leaves and kernels of the maize plant. Mutator genes also occur in micro-organisms including E. coli. As the action of mutator genes is directed at a particular

locus, they are said to produce directed mutations in contrast to random mutations which are not specific.

Normally all genes could mutate. In the case of a rare allele its mutation to other alleles is difficult to detect due to low frequency and slow rate of mutation. But when an allele occurs more frequently in the population it leads to higher mutation rate and increases the population's potential for evolutionary change. If a rare deleterious allele accumulates in the population, it is a disadvantage and constitutes what is referred to as the mutational load.

3. Selection:

Selection is one of the forces that change gene frequencies in the population and a fundamental process of evolutionary change. The idea was first conceived by Charles Darwin in his Origin of Species published in 1859 and by Alfred Russel Wallace.

Selection is defined as differential survival or fertility of different genotypes. If individuals carrying gene A are more successful in reproduction than individuals carrying its allele a, then the frequency of gene A will tend to be greater than that of gene a.

The wide variety of mechanisms responsible for modifying the reproductive success of a genotype are collectively included under selection. It is the process that determines the contribution that people of different genotypes will make as parents of the next generation. Selection does not act on individual genes, but rather on the organism bearing the genes.

The reproductive efficiency of a genotype is measured in terms of the average number of offspring born to the bearers of the genotype and is called Darwinian fitness or relative fitness. It is also referred to as the organism's adaptive value. The fitness value of 1 is usually assigned to the genotype with highest reproductive efficiency.

However, fitness does not have an absolute value, and is expressed in relative terms as a ratio. Relative fitness (w) is obtained by dividing the fitness of all the genotypes by the fitness of any one genotype. Fitness simply describes the average number of progeny that survive and reproduce. The related term selection coefficient $s = 1 – w$. Some aspects in the individual's life are likely to affect the survival, growth and reproduction; consequently they affect the fitness of genotypes and are referred to as fitness components.

Basically fitness depends upon survival and fertility. Persons affected with Huntington's chorea, a dominant condition may have 25% reproductive efficiency as compared to normal human beings. On the other hand children with Tay Sachs disease usually die before reproductive age. Thus the fitness of a person with Huntington's chorea is 0.25 and of Tay Sachs patient is zero.

When fitness of two alleles at a locus differs then selection favours survival of alleles with greater fitness and elimination of the other alleles. Thus frequency of one allele increases and of the other will decrease in the subsequent generations. However, if a rare allele occurs with low frequency, then selection is not able to cause much change in gene frequency.

Specifically selection occurs against a recessive allele, or a dominant allele, resulting in its elimination; it could occur in favour of a heterozygote or against a heterozygote leading to polymorphism in a given trait. When selection occurs in favour of a heterozygote over both homozygotes it is called over dominance or heterosis. It occurs when the fitness of the heterozygous genotype is greater than the fitness of both homozygotes. Assume that the relative fitness of the genotypes AA, Aa and aa are 0.9,1 and 0.8 respectively.

The greater fitness of the Aa- genotype will not allow either A or a alleles from homozygotes to become fixed. Ultimately equilibrium gene frequencies would be attained. In humans over dominance has led to polymorphism in sickle cell trait, thalassaemia and G6PD. The effect of selection is also counterbalanced by mutation. While selection is eliminating some genes from the population, mutation is creating new ones. The two forces selection and mutation operate in opposite directions, and tend to compensate each other. After a long time, gene frequencies will reach equilibrium.

There could be partial selection against recessives. This is a less complete form of selection against homozygous recessive individuals. In this case selection coefficient s is less than one, and the relative fitness w of the homozygous recessive individual is 1 – s, having value greater than zero. A popular example of selection is industrial melanism as exhibited by the pepper moth **Biston betularia**. In the mid19th century the light coloured forms of the moth were abundant on the pale barks of trees growing in unpolluted, non-industrialised regions of England. The dark form of Biston was extremely rare. As industry developed in the area, the environment became polluted and the barks of trees turned dark grey with smoke and dust.
The light moths on the dark coloured bark were easily noticed by the predators and were preyed upon. Their number began to decrease. In the following decades, the population of dark moths was observed to gradually increase to more than 95%; the light moths were hardly seen. Industrial melanism is thus a clear cut example of selection disturbing gene frequencies in the population.

Artificial and Natural Selection:
Selection was being practiced by humans since antiquity. Plant and animal breeders have been attempting to modify hereditary transmission of traits by selecting most desirable individuals to serve as parents for the next generation. This is called artificial selection. By contrast, when organisms are selected by natural forces instead of by human choice, they are said to be subject to natural selection.

4. Random Genetic Drift:
These are unexpected random changes that occur in gene frequencies from generation to generation in all populations. They are particularly noticeable as sampling variation in small populations. In some generations the frequency of a certain allele will by chance increase, in others it will decrease, in still others it may remain the same. These fluctuations in gene frequency occur at random. In small samples there is greater variation as compared to big samples. Drift however, does not depend upon the total size of the population, rather on the number of breeding individuals who would produce the next generation. It is unlikely that random drift alone will affect allelic frequencies at a gene locus over long periods of time. It is more likely that selection, mutation or migration would also take place at one time or another.

Measurement of Genotype Frequency:

One way of measuring genotype frequency is from phenotype frequency. Consider the case of 3 blood groups A, AB and B determined by two alleles I^A and I^B at a single locus. In a random sample of 1000 humans, the A group occurred in 210, AB in 450 and B in 340 individuals.
The frequencies of the blood group phenotypes and their respective genotypes are obtained by dividing the number of individuals for each blood group by the total. Thus, the frequency of blood group B for instance would be 340/1000 = 0.34.
Another way of estimating genotype frequency is to first calculate gene frequency of genes A and B in the population. Assume that the above sample contains 210 AA, 450 AB and 340 BB individuals.
The gene frequency of A in the population is represented by the probability to find A allele at the AB locus and is exactly equivalent to the proportion of A alleles among all alleles at this locus in the sample or in the population (that is because we cannot determine the frequency of A in the whole population).
As each individual carries two alleles at the AB locus, the total number of alleles in the sample is 1000 x 2 = 2000. Out of these 210 + 210 + 450 = 870 are A. Therefore the frequency of the A allele is 870/2000 = 0.435. The number of B alleles is 450 + 340 + 340 = 1130, and the frequency of B allele is 1130/2000 = 0.565. If we represent the gene frequency of A by p, then p represents a value between 0 and 1 (because the proportion of allele A must lie between 0 and 100 per cent). In our example p = 0.435. Similarly, if we symbolize the frequency of B by q, then q = 0.565. It may be noted that q = 1-p or 1- 0.435. Similarly p = 1 – q or 0.565. Thus p + q = 1.
For predicting genotype frequencies some assumptions have to be made, such as random mating in the population. That is to say, with respect to the trait of blood groups, an individual will mate with another without regard to whether the blood group of the mate is AA, AB or BB. Random mating implies random union of eggs and sperm, which in the example cited is the frequency of A and B alleles among the eggs and sperm (the gametes). Now the probability for the allele A at the AB locus in the population is p, and this is also the probability for a randomly chosen gamete to carry allele A. Similarly the probability for a randomly chosen gamete to carry B is q. For producing individuals with genotype AA, an A sperm must fertilize an A egg.
This occurs with probability p x p =p^2. An AB genotype results from fertilisation of A sperm with B egg or vice versa, and the probability is p x q or pq + qp = 2pq (that is pq for AB genotype and qp for BA genotype). The frequency of BB genotypes depends upon the chance fertilisation of a B sperm and B egg; this has probability q x q = q^2. Thus the frequencies of AA, AB and BB genotypes in the population should be expected to be p^2, 2pq, and q^2 respectively. If we substitute the values of p = 0.435 and q = 0.565 we can know that the frequency of AA = $(0.435)^2$ = 0.189, AB = 2 x 0.435 x 0.565 = 0.246, and of BB = $(0.565)^2$ = 0.319.

Significance of Population Genetics:

1. Knowledge of gene and genotype frequency in a population is useful for a plant breeder in the assessment of competitive ability of various genotypes in varietal mixtures. Such studies

help in identification of genotypes with high adaptive value. If such studies are conducted over multiplications, the varietal flexibility or stability can also be assessed in varietal blends. Hardy-Weinberg Law operates in random mating or panmictic species.
2. Study of gene frequency in a population also reveals significance of various factors in natural evolution. In cross pollinated crops, development of composite and synthetic varieties is based on Hardy-Weinberg principle.

Probable questions:

1. What is gene pool? How it is measured?
2. What happens if the gene pool gets smaller?
3. Explain classical hypothesis regarding population structure.
4.. Explain balance hypothesis regarding population structure.
5. State Hardy Weinberg's Law.
6. Define gene frequency. How it is calculated ?
7. Define genotype frequency. How it is calculated ?
8. What are assumptions of Hardy Weinberg's law. Explain.
9. What are the applications of Hardy Weinberg's law ?
10. How mutation disrupt Hardy Weinberg's law ?
11. How natural selection disrupts Hardy Weinberg's law ?
12. How migration disrupts Hardy Weinberg's law ?
13. What are the significance of population genetics ?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.

DISCLAIMER: This Self Learning Material (SLM) has been compiled from various authentic books, Journals articles, e-journals and other web sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-IV

HARD CORE THEORY PAPER

Animal Behaviour and Vector Biology

ZHT-411

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

W.B., INDIA

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and coordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

HARD CORE THEORY PAPER (ZHT- 411)

Animal Behaviour and Vector Biology

Group A: Animal Behaviour

Module	Unit	Content	Credit	Class	Time (h)	Page No.
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	II	Learning and memory : Forms of learning, learning and habitat selection - migration, navigation and orientation		1	1	14-32
	III	Kinship: Relatedness, inclusive fitness? selfishness, altruism		1	1	33-43
	IV	Conflict: Sexual selection, aggression, competition dominance, Infanticide.		1	1	44-63
	V	Communications: Channels, functions, origin and modification of signal, signal receiving mechanism.		1	1	64-77
	VI	Evolution of feeding behaviour: optimal foraging theory		1	1	78-84

Unit-I

Introduction to animal behaviour: History, foundation, approaches and methods

Objective: In this unit you will know about history, foundation, approaches and methods of animal behaviour.

Introduction

Do the squirrels in your neighbourhood bury acorns underground? Does your cat start meowing around the time you usually feed her? Do you start hanging around the kitchen when it's close to dinnertime? If you've noticed any of these things, congratulations—you've made your first observations in behavioural biology! These are all examples of animal behaviours. Yep, you and I count as animals too. In fact, these behaviours are just a tiny sampling of the amazing and diverse behaviours we can see in nature.

We could ask what behaviour is used for, but it might be better to ask, what *isn't* it used for? Animals have behaviours for almost every imaginable aspect of life, from finding food to wooing mates, from fighting off rivals to raising offspring. Some of these behaviours are innate, or hardwired, in an organism's genes. For instance, this is true of the squirrel and its acorn. Other behaviours are learned, such as your tendency to hang around the kitchen at dinnertime or your ability to read the words on this screen.

In this section, we'll take a closer look at animal behaviour—how it's studied, how it can run the gamut from hardwired to learn, what are its approaches and methods.

Animal behaviour is the scientific study of the wild and wonderful ways in which animals interact with each other, with other living beings, and with the environment. It explores how animals relate to their physical environment as well as to other organisms, and includes topics such as how animals find and defend resources, avoid predators, choose mates, reproduce, and care for their young-ones.

What is behaviour?

Animal behaviour includes all the ways animals interact with other members of their species, with organisms of other species, and with their environment. Behaviour can also be defined more narrowly as a change in the activity of an organism in response to a *stimulus*, an external or internal cue or combination of cues. e.g., your dog might start drooling—a change in activity—in response to the sight of food—a stimulus.

Ethology is a field of basic biology, like ecology or genetics. It focuses on the behaviours of diverse organisms in their natural environment.

How to understand a behaviour?

Nikolaas (Niko) Tinbergen was a Dutch ornithologist, or bird biologist, who studied behaviour and is now considered one of the founders of the field of ethology. Based on his own research, Tinbergen proposed four basic questions helpful in understanding any animal behaviour. Let's look at these questions, using the production of song by the zebra finch—a common songbird—as an example.

1. Causation—what causes the behaviour?

What triggers the behaviour, and what body parts, functions, and molecules are involved in carrying it out? Example: Singing is triggered in zebra finches by social cues, such as the proximity of a potential mate, as well as the appropriate hormonal state. The ability to produce songs is influenced by male hormones and occurs mainly in male birds. Songs are produced when air flows from air sacs in the bronchi through an organ called the syrinx. Certain parts of the brain control song production and are well-developed in male zebra finches.

2. Development—how does the behaviour develop?

Is the behaviour present early in life? Does it change over the course of the organism's lifetime? What experiences are necessary for its development? Example: Young male zebra finches first listen to the songs of nearby males of their species, particularly their fathers. Then, they start to practice singing. By adulthood, male zebra finches have learned to produce their own songs, which are unique but often have similarities to those of their fathers. Once a finch has perfected its song, the song remains fixed for life (Fig 1).

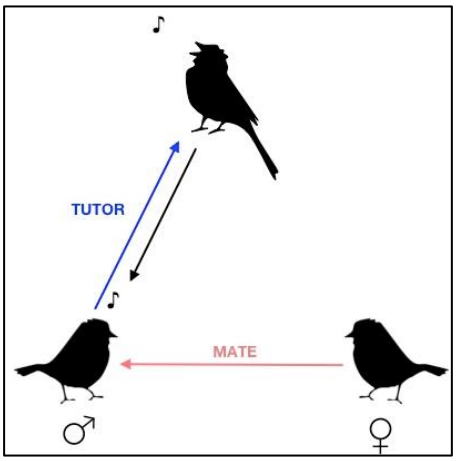

Fig 1: Learning behaviour of zebra finches

3. Function/adaptive value—how does the behaviour affect fitness?

How does the behaviour affect an organism's chances of survival and reproduction?

Example: Singing helps male zebra finches attract mates, increasing the chances that they will reproduce. Singing is part of an elaborate courtship ritual that entices the female to choose the male.

4. Phylogeny—how did the behaviour evolve?

How does the behaviour compare to those of related species? Why might it have evolved as it did?

Example: Almost all species of birds can make vocal sounds, but only those in the suborder *Passeri* are songbirds. Relative to the zebra finch, other songbird species differ in the timing of their listening and practicing phases, the plasticity of song over their lifetimes, the extent to which the song is similar among individuals of the species, and the way that singing is used—for example, for defence of territory vs. courtship of mates.

Innate vs. learned behaviours

When we are trying to understand how a behaviour develops and how it arose evolutionarily, one important question is whether the behaviour is genetically pre-programmed or acquired through experience.

Let's consider some vocabulary:

- *Innate* behaviours are genetically hardwired and are inherited by an organism from its parents.
- *Learned* behaviours are not inherited. They develop during an organism's lifetime as the result of experience and environmental influence.

Behavioural biologists have found that many behaviours have both an innate and a learned component. So, it's generally most accurate for us to ask *to what extent* a behaviour is innate or learned.

Mostly innate behaviours

There are some examples of behaviours that are really and truly hardwired. These behaviours take place in a highly predictable way in response to the right stimulus, even if the organism has never before encountered that stimulus. For example, an adult salamander will swim perfectly if it's placed in water, even if it never saw water when it was young and has never watched another salamander swim. In this case, the behaviour of swimming can only be explained as something genetically pre-programmed in the salamander. Similarly, you—or any human—will rapidly jerk your hand away if you touch a very hot object. This response is a reflex that's hardwired in the circuits of your sensory and motor neurons and doesn't even involve your brain.

Partly innate, partly learned behaviours

In other cases, an organism is genetically programmed to develop a behaviour, but the form the behaviour takes depends on the individual's experience. One example is the learning of a song by a zebra finch or other songbird, as we saw above. All male zebra finches will begin listening to and learning song at about the same age and practicing and producing song at a slightly later age. Although this pattern is genetically determined, the exact features of the song a bird sings will depend on the songs it hears during its learning period. Another, more familiar example is language acquisition in humans. Babies are pre-programmed for language learning, but which language they learn depends on what they're exposed to during their plastic, or formative, period.

Mostly learned behaviours

In other cases, behaviours are largely dependent on experience—they're learned—and can't be fully explained by genetic pre-programming. For instance, if a rat receives a food reward each time it pushes a lever, it will quickly learn to push the lever in order to get the food. Similarly, if a cow gets an electric shock each time it brushes up against an electric fence, like the one below, it will rapidly learn to avoid the fence. Pushing a lever to get a reward and avoiding electric fences are not hardwired in rats and cows but are, instead, learned behaviours the animals develop through experience.

If a behaviour is learned rather than innate, it isn't directly inherited. But it does still depend on genes. For instance, not all types of animals could learn to push a lever to get a reward. The rat's capacity to learn this behaviour depends on how its brain is wired, and the construction, maintenance, and function of a rat brain are all determined by genes in the rat genome.

Natural selection shapes behaviour

To the extent that a behaviour is genetically determined or relies on genes, it’s subject to evolutionary forces, such as natural selection. In many cases, we can see how a behaviour gives a survival or reproduction benefit to an animal that performs it—in other words, the behaviour increases fitness.

What are natural selection and fitness?

Here are some examples of behaviours that clearly increase fitness:

- Baby birds of many species instinctively open their mouths for food when the mother returns to the nest. Birds with this heritable behaviour will tend to get fed more—and thus survive to adulthood more—than those that don't.
- Mother greylag geese (Fig 2) instinctively roll eggs back into the nest if they fall out. Geese with this heritable behaviour will tend to have more offspring that survive to hatch than geese without the behaviour.

Fig 2: Greylag geese

- Zebra finch males learn songs while they are juveniles, young birds, and they use these songs in courtship rituals. Birds with the heritable tendency to learn a song will obtain a mate more often than those that don't.

An important point from the last example is that natural selection can act even when the behaviour itself is not inherited. A zebra finch doesn't inherit its song directly—it has to learn the song. But its capacity and tendency to learn a song are genetically determined, so they can be subject to natural selection.

Historical Perception in Animal Behaviour

During the course of history, interest in animal behaviour has stemmed from the fact that animals were a primary source of food, clothing, and materials for tools and shelter. Knowledge about their behaviour was, thus, necessary for successful hunting.
Moreover, there has always been human curiosity about the natural world. The historical perception, thus, relates from the early days of human existence to the present experimental and theoretical approaches.

A. Early Days of Human Existence:

Humans from early days were hunters and meat-eaters. The early hominids (*Homo erectus*) practiced crude variety of hunting. The Peking man (a form of *Homo erectus*), some 4, 00,000 years ago, was a better hunter. He knew the use of fire and made tools from animal bones. Early *Homo sapiens* were keen observers of animal behaviour and utilized this knowledge for hunting and evading predators. Hunters the cliff where they could be slaughtered with rocks or clubs. Cave paintings of prehistoric man suggest close observation of the animals at various times in their life cycles, from game to hunting, and, thus, depicting man's relationship with animals.

Later world:

After civilization, interest in animal behaviour increased due to curiosity about natural phenomenon and a desire to record and categories observations. Early scholars [Aristotle (384-322 B.C.), Pliny (23-79 A.D.) and others] attempted to record what they observed in the world around them.

However, their perception of animal behaviour was poor due to lack of full knowledge about what was taking place or due to biased religious or philosophical outlook. However, these formed the basis of later development in the field.

B. Emergence of Animal Behaviour as a Scientific Discipline:

Scientific study of animal behaviour emerged in the latter part of the nineteenth century.

Three major developments contributed significantly to the study of behaviour:

(a) Theory of evolution by natural selection,

(b) Development of a systematic comparative method, and

(c) Studies in genetics and inheritance.

(a) Darwin-Wallace theory of natural selection:

During the nineteenth century several voyages were made by Europeans to all parts of the globe for exploration and discovery of exotic fauna and flora. These scientists made observations and brought live and preserved specimens to zoos and laboratories in Europe, where scholars could observe, record their behaviour and interrelationships of these discovered species.

One such scientist was Charles Darwin (1809-1882) who made voyages to the Galapagos Islands. Darwin's thinking about the competition for survival among members of a species was influenced by Thomas Malthus' "Essay on the Principle of Population". Sir Charles Lyell (1797-1875), a geologist, made observations of rock strata and succession of fossils. This led others to believe that species were not fixed entities.

A. R. Wallace's (1823-1913) voyage to the Malay Archipelago and Darwin's travels on the Beagle to South America and South Pacific, led each man – independently – to formulate the theory of evolution by natural selection.

Behaviour, morphology and physiology of animals were thought to be subjected to the effects of natural selection. The theory of evolution by natural selection have been modified subsequently due to the recent developments in biology, particularly genetics.

(b) Systematic comparative method:

The use of comparative method in studying animal behaviour is credited to George John Romanes (1848-1894). The comparative method involves the studying of animals to get insights into the behaviour of humans.

Romanes proposed that there is a continuity of mental processes from one species to another. He put forward that although humans can know only their own thoughts, they could infer the mental process of animals, including other people, from knowledges of their own.

Romanes suggested that a sequence would be constructed for the evolution of various emotional states in animals — worms, who exhibit only surprise and fear, were placed at the lowest scale; insects were capable of various social feelings and curiosity; fishes showed play, jealousy and anger; reptiles exhibited affection; birds displayed pride and terror; and, finally, mammals were credited with hate, cruelty and shame. Romanes' theory relied largely on inferences rather than on recorded facts.

(c) Genetics and Inheritance:

Subsequently, the discovery of genetics and development of theories of inheritance by Gregor Mendel (1822-1884) greatly influenced research in animal behaviour. Present-day behavioral biology is a

combination of evolutionary theory (explains how traits change through time) and genetics (how traits are passed from generation to generation).
Any trait that evolves is passed on from generation to generation. Behaviour, thus, may change as a species evolves. Behaviour-genetic analysis from its early studies of inheritance got greatly expanded in the 1930s, and till modem times has been used as a powerful tool by many animal behaviourists.

C. Present Experimental and Theoretical Approaches:

The various theories, ideas etc. put forward in the latter half of the nineteenth century form the foundation of the present day's experimental approaches to the study of animal behaviour.

Four major approaches are given:

(a) Control mechanisms of behaviour has been sought through studies by comparative animal psychologists and physiologists. Much of the earlier psychological researches were heavily dependent on introspections and inferences. These methods have been later replaced by systematic, objective observations and replicable experiments.

Modern psychologists and physiologists explore areas such as learning processes, physiological control of behaviour, sensation and perception and behaviour genetics.

(b) The functional significance and evolution of behaviour patterns and explanations of behaviour mechanisms such as drives, innate releasing mechanisms etc. are the fields of classical ethologists. Behavioural traits are subject to natural selection.

Ethologists have traditionally made many of their research observations in a natural setting, conducted to assess the function of behaviour pattern. Ethological approach is used to determine how key stimuli trigger specific behaviour patterns. Modern ethology is concerned with four area of enquiry — causation, development, evolution, and function of behaviour.

(c) Environmental context for behaviour and the ways in which animals interact with their living and nonliving environments are under the preview of behavioural ecologists. Investigations are conducted in both field and laboratory settings.

(d) The study of the social behaviour and organization in animals are the fields of socio-biologists. Sociobiology has emerged as a new approach to the study of animal behaviour. It applies principles of evolutionary biology to the study of social behaviour in animals.

These varied approaches to the study of behaviour has led to the modern synthetic view of animals living and behaving in their natural environment. Although these approaches has been shown above as separate entities, they however, did not develop entirely independently of one another. In recent decades they have been moulded into a single discipline. The workers working in these fields may call themselves ethologists, animal behaviourists or comparative psychologists. They, however, are all pursuing the same goal using the same general theoretical frameworks and using similar experimental techniques and methods.

Aims and Objectives of Animal Behaviour:

Behaviour is recognized as one of the most important functions of animal life. The study of the behavior of animals is the final objective of all other branches of biology. Some of the elementary actions of the animals — to avoid predators, to gather food, to reproduce etc., depend on an extraordinary complex and beautiful synchronized mechanism of nerve cells, glands and muscles, along with a supporting skeleton.

These structures are part of a complex mechanism that must combine properly through the inherited constitution of each individual. Ethology has, thus, made important contributions to other disciplines like anthropology, sociology, psychology, physiology, environmental biology, sociobiology etc. Animal behaviour has also applications to human behaviour, to neurosciences, to the study of animal welfare and to the educating of future generations.

1. Ethology and Environment:

Animal behaviour generally provides the first clue to environmental degradation. Changes in sexual behaviour affects the population size of animals allowing us to take measures to save the environment. Field studies of natural behaviour of animals are vital to provide baseline data for environmental monitoring. For example, Environmental Protection Agencies use changes in swimming behaviour of minnows as an index of possible pesticide pollution.

Animal behaviour studies have led to the understanding of insect reproduction and host plant protection, leading to the discovery of non-toxic pheromones for insect pest control. Thus, the need for toxic pesticides can be avoided. Knowledge of predator-prey relationship would lead to the maintenance of proper food web in a given ecosystem.

2. Foraging Behaviour and Habitat Preservation:

Understanding of foraging behaviour in animals has led to an understanding of forest regeneration. Many animals serve as seed dispersers resulting in propagation of plant species and are, therefore, a tool for habitat preservation. Knowledge of foraging behaviour of honey bees, when applied to mechanisms of pollination can be important for plant breeding and propagation.

3. Animal Behaviour and Conservation:

It is essential that we know about the natural behaviour (foraging, reproductive, migratory, home range etc.) of endangered species in order to develop protective measures for their conservation. Re-introduction of animals into their natural habitat, such as the Golden lion tamarin of Brazil, requires detailed knowledge about the behaviour of such species.

Reproductive behaviour studies have led to improved captive breeding methods of near-extinct species such as snow leopard, red panda, golden lion tamarin, whooping cranes etc., so as to save them from extinction. Animal behaviour research, both in captivity and in natural habitat, has become increasingly important. Many of the world's leading conservationists have a background in animal behaviour or behavioural ecology.

4. Animal Behaviour and Economic Implication:

Research on salmon migration has revealed a lot about the mechanisms of migration. This information has been valuable in preserving the salmon industry in the Pacific Northwest, which has led to the development of the salmon fishing industry in the Great Lakes, USA.

Thus, basic animal behaviour research has important economic implications. Such behavioural researches, if conducted on hilsa fish, can result in a boom of the dwindling hilsa fishing industry in India.

5. Animal Behaviour and Welfare of Animals:

Knowledge about the welfare of animals is possible only through the studies of its behaviour. The Society of Animal Welfare has placed increased emphasis on the welfare of domestic, pet and research animals. Cruelty on animals is punishable by law. It is the duty of an ethologist to look at the behaviour and well-being of animals in laboratory and field.

6. Animal Behaviour and Neuro-ethology:

Through close observation of animal behaviour, Sir Charles Sherrington (1954), a Nobel Prize winner, developed a model for the structure and function of the nervous system. This work of Sherrington has been amply supported through subsequent neurobiological research on humans.

Neuro-ethology is the science of animal behaviour and neurobiology combined together. It provides an important framework for explaining neural mechanisms which can benefit humans. Behavioural studies of poisonous and venomous animals, and the extraction of poison (neurotoxic chemicals) from such animals, is used to make various medicines which has undoubtedly benefited mankind.

7. Animal Behaviour and Science Education:

Courses on animal behaviour and behavioural ecology have been recently introduced in Indian universities. Still, the related departments like Anthropology, Zoology, Psychology and Wildlife have very little syllabus on Ethology. Students are still far away from wildlife and their behaviour.

It is disheartening that most students have very little knowledge about the commonly found birds and animals around their own residence, leave aside those found in National Parks, Sanctuaries and Reserves. It is, therefore, essential that schools, colleges and universities should give emphasis on the study of Wildlife Biology, Conservation, Management, Animal Behaviour and Animal Husbandry.

8. Ethology and Human Behaviour:

Many problems in human society are related with the interaction of environment and animal behaviour. Studies on animal behaviour have led to interpretation of the framework of human society and to understand the various society-related problems.

Many studies on child abuses and infanticides in humans, finds its bearing from observation of animal behaviour. Various ethologists document that human societies have gradually evolved from animal societies and that understanding the behaviour of animals has helped in unfolding the mysteries of our own social organizations.

For example:

1. Researches on chimpanzee and monkey has illustrated the importance of cooperation and reconciliation in social groups. This work provides new dimensions for understanding the aggressive behaviour of human beings. The behavioural studies of human being would have been much less today without the influence of animal research.

2. Work on social development in rhesus monkeys has been of major importance to theories of child development and to psychiatry.

3. The woolly spider monkey in Brazil displays no aggressive behaviour among group members. Studies on how this species of monkey avoids aggression can be implemented to minimise human aggression.

4. Male parental care studies on California mouse, marmosets, tamarins and others can give us insight of father's involvement in child care.

5. Researches on circadian rhythms in animals has led to research relevant to human factors such as jet-lag or changing from one shift to another in an industry.

6. It has been possible to detect stress and psychological disorders through research on animal behaviour.

7. Researches on chimpanzee using language analogues have led to new technology (computer keyboards using arbitrary symbols) that has been successfully applied to teaching languages to disabled or physically challenged humans.

Probable Questions:

1. Define ethology.
2. Describe the steps of understanding the behaviour.
3. What do you mean by innate and learned behaviour?
4. Differentiate between innate and learned behaviour.
5. What is natural selection? How natural selection shapes behaviour?
6. Discuss the Historical Perception in Animal Behaviour
7. Discuss the aims of animal behaviour.

Suggested Readings:

1. Alcock, J. (2001). Animal Behaviour: An Evolutionary Approach. , Sinauer Associate Inc., USA.

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Unit-II

Learning and memory: Forms of learning, learning and habitat selection - migration, navigation and orientation

Objective: In this unit you will know about the basic idea of learning and memory; forms of learning, learning and habitat selection - migration, navigation and orientation.

Introduction

Memory is the faculty of the mind by which information is encoded, stored, and retrieved. Memory is vital to experiences and related to limbic systems, it is the retention of information over time for the purpose of influencing future action. If we could not remember past events, we could not learn or develop language, relationships, or personal identity.

Often memory is understood as an informational processing system with explicit and implicit functioning that is made up of a sensory processor, short-term (or working) memory, and long-term memory. This can be related to the neuron. The sensory processor allows information from the outside world to be sensed in the form of chemical and physical stimuli and attended to with various levels of focus and intent. Working memory serves as an encoding and retrieval processor. Information in the form of stimuli is encoded in accordance with explicit or implicit functions by the working memory processor. The working memory also retrieves information from previously stored material. Finally, the function of long-term memory is to store data through various categorical models or systems.

Explicit and implicit functions of memory are also known as declarative and non-declarative systems. These systems involve the purposeful intention of memory retrieval and storage, or lack thereof. Declarative, or explicit, memory is the conscious storage and recollection of data. Under declarative memory resides semantic and episodic memory. Semantic memory refers to memory that is encoded with specific meaning, while episodic memory refers to information that is encoded along a spatial and temporal plane. Declarative memory is usually the primary process thought of when referencing memory.

Non-declarative, or implicit, memory is the unconscious storage and recollection of information. An example of a non-declarative process would be the unconscious learning or retrieval of information by way of procedural memory, or a priming phenomenon. Priming is the process of subliminally arousing specific responses from memory and shows that not all memory is consciously activated, whereas procedural memory is the slow and gradual learning of skills that often occurs without conscious attention to learning.

Memory is not a perfect processor, and is affected by many factors. The manner information is encoded, stored, and retrieved can all be corrupted. The amount of attention given new stimuli can diminish the amount of information that becomes encoded for storage. Also, the storage process can become corrupted by physical damage to areas of the brain that are associated with memory storage, such as the hippocampus. Finally, the retrieval of information from long-term memory can be disrupted because of decay within long-term memory. Normal functioning, decay over time, and brain damage all affect the accuracy and capacity of memory. Memory loss is usually described as forgetfulness or amnesia.

Sensory memory

Sensory memory holds sensory information less than one second after an item is perceived. The ability to look at an item and remember what it looked like with just a split second of observation, or memorization, is the example of sensory memory. It is out of cognitive control and is an automatic response. With very short presentations, participants often report that they seem to "see" more than they can actually report. The first experiments exploring this form of sensory memory were precisely conducted by George Sperling (1963) using the "partial report paradigm". Subjects were presented with a grid of 12 letters, arranged into three rows of four. After a brief presentation, subjects were then played either a high, medium or low tone, cuing them which of the rows to report. Based on these partial report experiments, Sperling was able to show that the capacity of sensory memory was approximately 12 items, but that it degraded very quickly (within a few hundred milliseconds). Because this form of memory degrades so quickly, participants would see the display but be unable to report all of the items (12 in the "whole report" procedure) before they decayed. This type of memory cannot be prolonged via rehearsal.

Three types of sensory memories exist. Iconic memory is a fast decaying store of visual information; a type of sensory memory that briefly stores an image which has been perceived for a small duration. Echoic memory is a fast decaying store of auditory information, another type of sensory memory that briefly stores sounds that have been perceived for short durations. Haptic memory is a type of sensory memory that represents a database for touch stimuli.

Short-term memory

Short-term memory is also known as working memory. Short-term memory allows recall for a period of several seconds to a minute without rehearsal. Its capacity is also very limited: George A. Miller (1956), when working at Bell Laboratories, conducted experiments showing that the store of short-term memory was 7±2 items (the title of his famous paper, "The magical number 7±2"). Modern estimates of the capacity of short-term memory are lower, typically of the order of 4–5 items; however, memory capacity can be increased through a process called chunking. For example, in recalling a ten-digit telephone number, a person could chunk the digits into three groups: first, the area code (such as 123), then a three-digit chunk (456) and lastly a four-digit chunk (7890). This method of remembering telephone numbers is far more effective than attempting to remember a string of 10 digits; this is because we are able to chunk the information into meaningful groups of numbers. This may be reflected in some countries in the tendency to display telephone numbers as several chunks of two to four numbers.

Short-term memory is believed to rely mostly on an acoustic code for storing information, and to a lesser extent a visual code. Conrad (1964) found that test subjects had more difficulty recalling collections of letters that were acoustically similar (e.g. E, P, D). Confusion with recalling acoustically similar letters rather than visually similar letters implies that the letters were encoded acoustically. Conrad's (1964) study, however, deals with the encoding of written text; thus, while memory of written language may rely on acoustic components, generalisations to all forms of memory cannot be made.

Long term memory

The storage in sensory memory and short-term memory generally has a strictly limited capacity and duration, which means that information is not retained indefinitely. By contrast, long-term memory can store much larger quantities of information for potentially unlimited duration (sometimes a whole life span). Its capacity is immeasurable. For example, given a random seven-digit number we may remember it for only a few seconds before forgetting, suggesting it was stored in our short-term memory. On the other hand, we can remember telephone numbers for many years through repetition; this information is said to be stored in long-term memory.

While short-term memory encodes information acoustically, long-term memory encodes it semantically: Baddeley (1966) discovered that, after 20 minutes, test subjects had the most difficulty recalling a collection of words that had similar meanings (e.g. big, large, great, huge) long-term. Another part of long-term memory is episodic memory, "which attempts to capture information such as 'what', 'when' and 'where'". With episodic memory, individuals are able to recall specific events such as birthday parties and weddings.

Short-term memory is supported by transient patterns of neuronal communication, dependent on regions of the frontal lobe (especially dorsolateral prefrontal cortex) and the parietal lobe. Long-term memory, on the other hand, is maintained by more stable and permanent changes in neural connections widely spread throughout the brain. The hippocampus is essential (for learning new information) to the consolidation of information from short-term to long-term memory, although it does not seem to store information itself. It was thought that without the hippocampus new memories were unable to be stored into long-term memory and that there would be a very short attention span, as first gleaned from patient Henry Molaison after what was thought to be the full removal of both his hippocampi. More recent examination of his brain, post-mortem, shows that the hippocampus was more intact than first thought, throwing theories drawn from the initial data into question. The hippocampus may be involved in changing neural connections for a period of three months or more after the initial learning.

Research has suggested that long-term memory storage in humans may be maintained by DNA methylation, and the 'prion' gene.

Learning:

Learning is the process of acquiring new, or modifying existing, knowledge, behaviors, skills, values, or preferences. The ability to learn is possessed by humans, animals, and some machines; there is also evidence for some kind of learning in some plants. Some learning is immediate, induced by a single event (e.g. being burned by a hot stove), but much skill and knowledge accumulates from repeated experiences. The changes induced by learning often last a lifetime, and it is hard to distinguish learned material that seems to be "lost" from that which cannot be retrieved.

Human learning begins before birth and continues until death as a consequence of ongoing interactions between person and environment. The nature and processes involved in learning are studied in many fields, including educational psychology, neuropsychology, experimental, and pedagogy. Research in such fields has led to the identification of various sorts of learning. For example, learning may occur as a result of habituation, or classical conditioning, operant conditioning or as a result of more complex activities such as play, seen only in relatively intelligent animals. Learning may occur consciously or without conscious awareness. Learning that an aversive event can't be avoided nor escaped may result in a condition called learned helplessness. There is evidence for human behavioral learning prenatally, in which habituation has been observed as early as

32 weeks into gestation, indicating that the central nervous system is sufficiently developed and primed for learning and memory to occur very early on in development.

Play has been approached by several theorists as the first form of learning. Children experiment with the world, learn the rules, and learn to interact through play. Lev Vygotsky agrees that play is pivotal for children's development, since they make meaning of their environment through playing educational games.

Types

1. Non-associative learning

Non-associative learning refers to "a relatively permanent change in the strength of response to a single stimulus due to repeated exposure to that stimulus. Changes due to such factors as sensory adaptation, fatigue, or injury do not qualify as non-associative learning." Non-associative learning can be divided into habituation and sensitization.

a. Habituation

Habituation is an example of non-associative learning in which the strength or probability of a response diminishes when the stimulus is repeated. The response is typically a reflex or unconditioned response. Thus, habituation must be distinguished from extinction, which is an associative process. In operant extinction, for example, a response declines because it is no longer followed by reward. An example of habituation can be seen in small song birds—if a stuffed owl (or similar predator) is put into the cage, the birds initially react to it as though it were a real predator. Soon the birds react less, showing habituation. If another stuffed owl is introduced (or the same one removed and re-introduced), the birds react to it again as though it were a predator, demonstrating that it is only a very specific stimulus that is habituated to (namely, one particular unmoving owl in one place). Habituation has been shown in essentially every species of animal, as well as the sensitive plant *Mimosa pudica* and the large protozoan *Stentor coeruleus*.

b. Sensitization

Sensitization is an example of non-associative learning in which the progressive amplification of a response follows repeated administrations of a stimulus. An everyday example of this mechanism is the repeated tonic stimulation of peripheral nerves that occurs if a person rubs their arm continuously. After a while, this stimulation creates a warm sensation that eventually turns painful. The pain results from the progressively amplified synaptic response of the peripheral nerves warning that the stimulation is harmful. Sensitization is thought to underlie both adaptive as well as maladaptive learning processes in the organism.

2. Active learning

Experiential learning is more efficient than passive learning like reading or listening. Active learning occurs when a person takes control of his/her learning experience. Since understanding information is the key aspect of learning, it is important for learners to recognize what they understand and what they do not. By doing so, they can monitor their own mastery of subjects. Active learning encourages learners to have an internal dialogue in which they verbalize understandings. This and other meta-cognitive strategies can be taught to a child over time. Studies within metacognition have proven the value in active learning, claiming that the learning is usually at a stronger level as a result. In addition, learners have more incentive to learn when they have control over not only how they learn but also what they learn. Active learning is a key characteristic

of student-centred learning. Conversely, passive learning and direct instruction are characteristics of teacher-centred learning (or traditional education).

3. Associative learning

Associative learning is the process by which a person or animal learns an association between two stimuli. In classical conditioning a previously neutral stimulus is repeatedly paired with a reflex eliciting stimulus until eventually the neutral stimulus elicits a response on its own. In operant conditioning, a behavior that is reinforced or punished in the presence of a stimulus becomes more or less likely to occur in the presence of that stimulus.

4. Operant conditioning

In operant conditioning, a reinforcement (by reward) or instead a punishment given after a given behavior, change the frequency and/or form of that behavior. Stimulus present when the behavior/consequence occurs comes to control these behavior modifications.

5. Classical conditioning

The typical paradigm for classical conditioning involves repeatedly pairing an unconditioned stimulus (which unfailingly evokes a reflexive response) with another previously neutral stimulus (which does not normally evoke the response). Following conditioning, the response occurs both to the unconditioned stimulus and to the other, unrelated stimulus (now referred to as the "conditioned stimulus"). The response to the conditioned stimulus is termed a conditioned response. The classic example is Ivan Pavlov and his dogs. Pavlov fed his dogs meat powder, which naturally made the dogs salivate—salivating is a reflexive response to the meat powder. Meat powder is the unconditioned stimulus (US) and the salivation is the unconditioned response (UR). Pavlov rang a bell before presenting the meat powder. The first time Pavlov rang the bell, the neutral stimulus, the dogs did not salivate, but once he put the meat powder in their mouths they began to salivate. After numerous pairings of bell and food, the dogs learned that the bell signaled that food was about to come, and began to salivate when they heard the bell. Once this occurred, the bell became the conditioned stimulus (CS) and the salivation to the bell became the conditioned response (CR). Classical conditioning has been demonstrated in many species. For example, it is seen in honeybees, in the proboscis extension reflex paradigm. And recently, it was demonstrated in garden pea plants.

Another influential person in the world of classical conditioning is John B. Watson. Watson's work was very influential and paved the way for B.F. Skinner's radical behaviorism. Watson's behaviorism (and philosophy of science) stood in direct contrast to Freud and other accounts based largely on introspection. Watson's view was that the introspective method was too subjective, and that we should limit the study of human development to directly observable behaviors. In 1913, Watson published the article "Psychology as the Behaviorist Views," in which he argued that laboratory studies should serve psychology best as a science. Watson's most famous, and controversial, experiment, "Little Albert", where he demonstrated how psychologists can account for the learning of emotion through classical conditioning principles.

6. Observational Learning

Observational learning is learning that occurs through observing the behavior of others. It is a form of social learning which takes various forms, based on various processes. In humans, this form of learning seems to not need reinforcement to occur, but instead, requires a social model such as a parent, sibling, friend, or teacher with surroundings.

Imprinting

Imprinting is a kind of learning occurring at a particular life stage that is rapid and apparently independent of the consequences of behavior. In filial imprinting, young animals, particularly birds, form an association with another individual or in some cases, an object that they respond to as they would to a parent. In 1935, the Austrian Zoologist Konrad Lorenz discovered that certain birds follow and form a bond if the object makes sounds.

Play

Play generally describes behavior with no particular end in itself, but that improves performance in similar future situations. This is seen in a wide variety of vertebrates besides humans, but is mostly limited to mammals and birds. Cats are known to play with a ball of string when young, which gives them experience with catching prey. Besides inanimate objects, animals may play with other members of their own species or other animals, such as orcas playing with seals they have caught. Play involves a significant cost to animals, such as increased vulnerability to predators and the risk of injury and possibly infection. It also consumes energy, so there must be significant benefits associated with play for it to have evolved. Play is generally seen in younger animals, suggesting a link with learning. However, it may also have other benefits not associated directly with learning, for example improving physical fitness.

Play, as it pertains to humans as a form of learning is central to a child's learning and development. Through play, children learn social skills such as sharing and collaboration. Children develop emotional skills such as learning to deal with the emotion of anger, through play activities. As a form of learning, play also facilitates the development of thinking and language skills in children.

There are five types of play:

1. sensorimotor play aka functional play, characterized by repetition of activity
2. role play occurs starting at the age of 3
3. rule-based play where authoritative prescribed codes of conduct are primary
4. construction play involves experimentation and building
5. movement play aka physical play

These five types of play are often intersecting. All types of play generate thinking and problem-solving skills in children. Children learn to think creatively when they learn through play. Specific activities involved in each type of play change over time as humans progress through the lifespan. Play as a form of learning, can occur solitarily, or involve interacting with others.

Enculturation

Enculturation is the process by which people learn values and behaviors that are appropriate or necessary in their surrounding culture. Parents, other adults, and peers shape the individual's understanding of these values. If successful, enculturation results in competence in the language, values and rituals of the culture. This is different from acculturation, where a person adopts the values and societal rules of a culture different from their native one.

Multiple examples of enculturation can be found cross-culturally. Collaborative practices in the Mazahua people have shown that participation in everyday interaction and later learning activities

contributed to enculturation rooted in nonverbal social experience. As the children participated in everyday activities, they learned the cultural significance of these interactions. The collaborative and helpful behaviors exhibited by Mexican and Mexican-heritage children is a cultural practice known as being "acomedido". Chillihuani girls in Peru described themselves as weaving constantly, following behavior shown by the other adults.

- **Episodic learning**

Episodic learning is a change in behavior that occurs as a result of an event. For example, a fear of dogs that follows being bitten by a dog is episodic learning. Episodic learning is so named because events are recorded into episodic memory, which is one of the three forms of explicit learning and retrieval, along with perceptual memory and semantic memory.

- **Multimedia learning**

Multimedia learning is where a person uses both auditory and visual stimuli to learn information. This type of learning relies on dual-coding theory.

- **E-learning and augmented learning**

Electronic learning or e-learning is computer-enhanced learning. A specific and always more diffused e-learning is mobile learning (m-learning), which uses different mobile telecommunication equipment, such as cellular phones.

When a learner interacts with the e-learning environment, it's called augmented learning. By adapting to the needs of individuals, the context-driven instruction can be dynamically tailored to the learner's natural environment. Augmented digital content may include text, images, video, audio (music and voice). By personalizing instruction, augmented learning has been shown to improve learning performance for a lifetime. See also minimally invasive education.

Moore (1989) reported that three core types of interaction are necessary for quality, effective online learning:

→ Learner–learner (i.e. communication between and among peers with or without the teacher present),

→ Learner–instructor (i.e. student teacher communication), and

→ Learner–content (i.e. intellectually interacting with content that results in changes in learners' understanding, perceptions, and cognitive structures).

In his theory of transactional distance, Moore (1993) contented that structure and interaction or dialogue bridge the gap in understanding and communication that is created by geographical distances (known as transactional distance).

- **Rote learning**

Rote learning is memorizing information so that it can be recalled by the learner exactly the way it was read or heard. The major technique used for rote learning is learning by repetition, based on the idea that a learner can recall the material exactly (but not its meaning) if the information is repeatedly processed. Rote learning is used in diverse areas, from mathematics to music to religion. Although it has been criticized by some educators, rote learning is a necessary precursor to meaningful learning.

- **Meaningful learning**

Meaningful learning is the concept that learned knowledge (e.g., a fact) is fully understood to the extent that it relates to other knowledge. To this end, meaningful learning contrasts with rote learning in which information is acquired without regard to understanding. Meaningful learning, on the other hand, implies there is a comprehensive knowledge of the context of the facts learned.

- **Informal learning**

Informal learning occurs through the experience of day-to-day situations (for example, one would learn to look ahead while walking because of the danger inherent in not paying attention to where one is going). It is learning from life, during a meal at table with parents, play, exploring, etc.

- **Formal learning**

Formal learning is learning that takes place within a teacher-student relationship, such as in a school system. The term formal learning has nothing to do with the formality of the learning, but rather the way it is directed and organized. In formal learning, the learning or training departments set out the goals and objectives of the learning.

- **Non-formula learning**

Non-formal learning is organized learning outside the formal learning system. For example, learning by coming together with people with similar interests and exchanging viewpoints, in clubs or in (international) youth organizations, workshops.

Non-formal learning and combined approaches

The educational system may use a combination of formal, informal, and non-formal learning methods. The UN and EU recognize these different forms of learning. In some schools, students can get points that count in the formal-learning systems if they get work done in informal-learning circuits. They may be given time to assist international youth workshops and training courses, on the condition they prepare, contribute, share and can prove this offered valuable new insight, helped to acquire new skills, a place to get experience in organizing, teaching, etc. To learn a skill, such as solving a Rubik's Cube quickly, several factors come into play at once:

- ✓ Reading directions helps a player learn the patterns that solve the Rubik's Cube.
- ✓ Practicing the moves repeatedly helps build "muscle memory" and speed.
- ✓ Thinking critically about moves helps find shortcuts, which speeds future attempts.
- ✓ Observing the Rubik's Cube's six colours help anchor solutions in the mind.
- ✓ Revisiting the cube occasionally helps retain the skill.

- **Tangential learning**

Tangential learning is the process by which people self-educate if a topic is exposed to them in a context that they already enjoy. For example, after playing a music-based video game, some people may be motivated to learn how to play a real instrument, or after watching a TV show that references Faust and Lovecraft, some people may be inspired to read the original work. Self-education can be improved with systematization. According to experts in natural learning, self-oriented learning training has proven an effective tool for assisting independent learners with the natural phases of learning.

Extra Credits writer and game designer James Portnow was the first to suggest games as a potential venue for "tangential learning". Mozelius et al. points out that intrinsic integration of learning content seems to be a crucial design factor, and that games that include modules for further self-studies tend to present good results. The built-in encyclopedias in the Civilization games are presented as an example - by using these modules gamers can dig deeper for knowledge about historical events in the gameplay. The importance of rules that regulate learning modules and game experience is discussed by Moreno, C., in a case study about the mobile game. In this game, developed by Landka in collaboration with ESA and ESO, game progress is rewarded with educational content, as opposed to traditionaleducation games where learning activities are rewarded with gameplay.

- **Dialogic learning**

Dialogic learning is a type of learning based on dialogue.

- **Incidental learning**

In incidental teaching learning is not planned by the instructor or the student, it occurs as a byproduct of another activity — an experience, observation, self-reflection, interaction, unique event, or common routine task. This learning happens in addition to or apart from the instructor's plans and the student's expectations. An example of incidental teaching is when the instructor places a train set on top of a cabinet. If the child points or walks towards the cabinet, the instructor prompts the student to say "train." Once the student says "train," he gets access to the train set.

Here are some steps most commonly used in incidental teaching:

- ✓ An instructor will arrange the learning environment so that necessary materials are within the student's sight, but not within his reach, thus impacting his motivation to seek out those materials.
- ✓ An instructor waits for the student to initiate engagement.
- ✓ An instructor prompts the student to respond if needed.
- ✓ An instructor allows access to an item/activity contingent on a correct response from the student.
- ✓ The instructor fades out the prompting process over a period of time and subsequent trials.

Incidental learning is an occurrence that is not generally accounted for using the traditional methods of instructional objectives and outcomes assessment. This type of learning occurs in part as a product of social interaction and active involvement in both online and onsite courses. Research implies that some un-assessed aspects of onsite and online learning challenge the equivalency of education between the two modalities. Both onsite and online learning have distinct advantages with traditional on-campus students experiencing higher degrees of incidental learning in three times as many areas as online students. Additional research is called for to investigate the implications of these findings both conceptually and pedagogically.

- **Machine learning**

Machine learning, a branch of artificial intelligence, concerns the construction and study of systems that can learn from data. For example, a machine learning system could be trained on email messages to learn to distinguish between spam and non-spam messages.

Domains

Benjamin Bloom has suggested three domains of learning:

→ *Cognitive*: To recall, calculate, discuss, analyze, problem solve, etc.

→ *Psychomotor*: To dance, swim, ski, dive, drive a car, ride a bike, etc.

→ *Affective*: To like something or someone, love, appreciate, fear, hate, worship, etc.

These domains are not mutually exclusive. For example, in learning to playchess, the person must learn the rules (cognitive domain)—but must also learn how to set up the chess pieces and how to properly hold and move a chess piece (psychomotor). Furthermore, later in the game the person may even learn to love the game itself, value its applications in life, and appreciate its history (affective domain).

Transfer

Transfer of learning is the application of skill, knowledge or understanding to resolve a novel problem or situation that happens when certain conditions are fulfilled. Research indicates that learning transfer is infrequent; most common when "... cued, primed, and guided..." and has sought to clarify what it is, and how it might be promoted through instruction.

Over the history of its discourse, various hypotheses and definitions have been advanced. First, it is speculated that different types of transfer exist, including: near transfer, the application of skill to solve a novel problem in a similar context; and far transfer, the application of skill to solve novel problem presented in a different context. Furthermore, Perkins and Salomon (1992) suggest that positive transfer in cases when learning supports novel problem solving, and negative transfer occurs when prior learning inhibits performance on highly correlated tasks, such as second or third-language learning. Concepts of positive and negative transfer have a long history; researchers in the early 20th century described the possibility that "...habits or mental acts developed by a particular kind of training may inhibit rather than facilitate other mental activities". Finally, Schwarz, Bransford and Sears (2005) have proposed that transferring knowledge into a situation may differ from transferring knowledge out to a situation as a means to reconcile findings that transfer may both be frequent and challenging to promote.

A significant and long research history has also attempted to explicate the conditions under which transfer of learning might occur. Early research by Ruger, for example, found that the "level of attention", "attitudes", "method of attack" (or method for tackling a problem), a "search for new points of view", "a careful testing of hypothesis" and "generalization" were all valuable approaches for promoting transfer. To encourage transfer through teaching, Perkins and Salomon recommend aligning ("hugging") instruction with practice and assessment, and "bridging", or encouraging learners to reflect on past experiences or make connections between prior knowledge and current content.

Factors affecting learning

External factors

1. Heredity: A classroom instructor can neither change nor increase heredity, but the student can use and develop it. Some learners are rich in hereditary endowment while others are poor. Each student is unique and has different abilities. The native intelligence is different in individuals. Heredity governs or conditions our ability to learn and the rate of learning. The intelligent learners can establish and see relationship very easily and more quickly.

2. Status of students: Physical and home conditions also matter: Certain problems like malnutrition i.e.; inadequate supply of nutrients to the body, fatigue i.e.; tiredness, bodily weakness, and bad health are great obstructers in learning. These are some of the physical conditions by which a student can get affected. Home is a place where a family lives. If the home conditions are not proper, the student is affected seriously. Some of the home conditions are bad ventilation, unhygienic living, bad light, etc. These affect the student and his or her rate of learning.

3. Physical environment: The design, quality, and setting of a learning space, such as a school or classroom, can each be critical to the success of a learning environment. Size, configuration, comfort—fresh air, temperature, light, acoustics, furniture—can all affect a student's learning. The tools used by both instructors and students directly affect how information is conveyed, from display and writing surfaces (blackboards, markerboards, tack surfaces) to digital technologies. For example, if a room is too crowded, stress levels rise, student attention is reduced, and furniture arrangement is restricted. If furniture is incorrectly arranged, sight lines to the instructor or instructional material is limited and the ability to suit the learning or lesson style is restricted. Aesthetics can also play a role, for if student morale suffers, so does motivation to attend school.

Internal factors

There are several internal factors that affect learning. They are

***1. Goals or purposes*:** Each and everyone has a goal. A goal should be set to each pupil according to the standard expected to him. A goal is an aim or desired result. There are 2 types of goals called immediate and distant goals. A goal that occurs or is done at once is called an immediate goal, and distant goals are those that take time to achieve. Immediate goals should be set before the young learner and distant goals for older learners. Goals should be specific and clear, so that learners understand.

2. Motivational behaviour: Motivation means to provide with a motive. Motivation learners should be motivated so that they stimulate themselves with interest. This behaviour arouses and regulates the student's internal energies.

3. Interest: This is a quality that arouses a feeling. It encourages a student to move over tasks further. During teaching, the instructor must raise interests among students for the best learning. Interest is an apparent (clearly seen or understood) behaviour.

4. Attention: Attention means consideration. It is concentration or focusing of consciousness upon one object or an idea. If effective learning should take place attention is essential. Instructors must secure the attention of the student.

5. Drill or practice: This method includes repeating the tasks "n" number of times like needs, phrases, principles, etc. This makes learning more effective.

6. Fatigue: Generally there are three types of fatigue, i.e., muscular, sensory, and mental. Muscular and sensory fatigues are bodily fatigue. Mental fatigue is in the central nervous system. The remedy is to change teaching methods, e.g., use audio-visual aids, etc.

7. Aptitude: Aptitude is natural ability. It is a condition in which an individual’s ability to acquire certain skills, knowledge through training.

8. Attitude: It is a way of thinking. The attitude of the student must be tested to find out how much inclination he or she has for learning a subject or topic.

9. Emotional conditions: Emotions are physiological states of being. Students who answer a question properly or give good results should be praised. This encouragement increases their ability

and helps them produce better results. Certain attitudes, such as always finding fault in a student's answer or provoking or embarrassing the student in front of a class are counterproductive.

***10. Speed, Accuracy and retention*:** Speed is the rapidity of movement. Retention is the act of retaining. These 3 elements depend upon aptitude, attitude, interest, attention and motivation of the students.

11. Learning activities: Learning depends upon the activities and experiences provided by the teacher, his concept of discipline, methods of teaching and above all his overall personality.

12. Testing: Various tests measure individual learner differences at the heart of effective learning. Testing helps eliminate subjective elements of measuring pupil differences and performances.

***13. Guidance*:** Everyone needs guidance in some part or some time in life. Some need it constantly and some very rarely depending on the students' conditions. Small learners need more guidance. Guidance is an advice to solve a problem. Guidance involves the art of helping boys and girls in various aspects of academics, improving vocational aspects like choosing careers and recreational aspects like choosing hobbies. Guidance covers the whole gamut of learners' problems- learning as well as non- learning.

In animal evolution

Animals gain knowledge in two ways. First is learning—in which an animal gathers information about its environment and uses this information. For example, if an animal eats something that hurts its stomach, it learns not to eat that again. The second is innate knowledge that is genetically inherited.

An example of this is when a horse is born and can immediately walk. The horse has not learned this behavior; it simply knows how to do it. In some scenarios, innate knowledge is more beneficial than learned knowledge. However, in other scenarios the opposite is true—animals must learn certain behaviors when it is disadvantageous to have a specific innate behavior. In these situations, learning evolves in the species.

Costs and benefits of learned and innate knowledge

In a changing environment, an animal must constantly gain new information to survive. However, in a stable environment, this same individual needs to gather the information it needs once, and then rely on it for the rest of its life. Therefore, different scenarios better suit either learning or innate knowledge. Essentially, the cost of obtaining certain knowledge versus the benefit of already having it determines whether an animal evolved to learn in a given situation, or whether it innately knew the information. If the cost of gaining the knowledge outweighs the benefit of having it, then the animal does not evolve to learn in this scenario—but instead, non-learning evolves. However, if the benefit of having certain information outweighs the cost of obtaining it, then the animal is far more likely to evolve to have to learn this information.

Non-learning is more likely to evolve in two scenarios. If an environment is static and change does not or rarely occurs, then learning is simply unnecessary. Because there is no need for learning in this scenario—and because learning could prove disadvantageous due to the time it took to learn the information—non-learning evolves. However, if an environment is in a constant state of change, then learning is disadvantageous. Anything learned is immediately irrelevant because of the changing environment. The learned information no longer applies. Essentially, the animal would be just as successful if it took a guess as if it learned. In this situation, non-learning evolves. In fact, a study

of *Drosophila melanogaster* showed that learning can actually lead to a decrease in productivity, possibly because egg-laying behaviors and decisions were impaired by interference from the memories gained from the new learned materials or because of the cost of energy in learning.

However, in environments where change occurs within an animal's lifetime but is not constant, learning is more likely to evolve. Learning is beneficial in these scenarios because an animal can adapt to the new situation, but can still apply the knowledge that it learns for a somewhat extended period of time. Therefore, learning increases the chances of success as opposed to guessing. An example of this is seen in aquatic environments with landscapes subject to change. In these environments, learning is favored because the fish are predisposed to learn the specific spatial cues where they live.

Habitat

In ecology, a habitat is the type of natural environment in which a particular species of organism lives. A species' habitat is those places where the species can find food, shelter, protection and mates for reproduction. It is characterized by both physical and biological features.

The physical factors may include (for example): soil, moisture, range of temperature, and light intensity. Biotic factors will include the availability of food and the presence or absence of predators. Every organism has certain habitat needs for the conditions in which it will thrive, but some are tolerant of wide variations while others are very specific in their requirements. A habitat is not necessarily a geographical area, it can be the interior of a stem, a rotten log, a rock or a clump of moss; for a parasitic organism has as its habitat the body of its host, part of the host's body (such as the digestive tract), or a single cell within the host's body.

Geographic habitat types include polar, temperate, subtropical and tropical. The terrestrial vegetation type maybe forest, steppe and grassland, semi-arid or desert. Freshwater habitats include marshes, streams, rivers, lakes, and ponds; marine habitats include salt marshes, the coast, the intertidal zone, estuaries, reefs, bays, the open sea, the sea bed, deep water and submarine vents.

Habitats may change over time. Causes of change may include a violent event (such as the eruption of a volcano, an earthquake, a tsunami, a wildfire or a change in oceanic currents); or change may occur more gradually over millennia with alterations in the climate, as ice sheets and glaciers advance and retreat, and as different weather patterns bring changes of precipitation and solar radiation. Other changes come as a direct result of human activities, such as deforestation, the ploughing of ancient grasslands, the diversion and damming of rivers, the draining of marshland and the dredging of the seabed. The introduction of alien species can have a devastating effect on native wildlife, through increased predation, through competition for resources or through the introduction of pests and diseases to which the indigenous species have no immunity.

Habitat selection

Habitat selection is a hierarchical process involving a series of innate and learned behavioural decisions made by an animal about what habitat it would use at different scales of the environment Studies of habitat selection by deer mice (*Peromyscus maniculatus*) revealed that heredity and experience play a role in determining selection. Habitat selection was generated by foraging decisions. However, foraging is only one behaviour driving habitat selection.

Habitat may be selected for cover availability, forage quality and quantity, and resting or denning sites. Proximate factors serve as cues an animal uses to determine the suitability of a site including the specific vegetation composition within a desired habitat. Reproductive success and survival of the

species are the ultimate reasons that influence a species to select a habitat. The ability to persist is governed by ultimate factors such as forage availability, shelter, and avoiding predators. Several interacting factors have an influence on habitat selection for an individual (e.g., competition, cover, and predation).

Competition is involved because each individual is involved in intraspecific and interspecific relationships that partition the available resources within an environment. Competition may result in a species failing to select a habitat suitable in all other resources or may determine spatial distribution within the habitat (Keen 1982). Predation also complicates selection of habitat. The existence of predators may prevent an individual from occupying an area. Survival of the species and its future reproductive success are the driving forces that presumably cause an individual to evaluate these biotic factors. With a high occurrence of competition and predators, an individual may choose a different site with less optimal resources. Once predators are removed, areas with necessary resources can then be inhabited. Habitat selection is therefore an active behavioural process by an animal. Each species searches for features within an environment that are directly or indirectly associated with the resources that an animal would need to reproduce, survive, and persist. Habitat selection is a compilation of innate and learned behaviours that lie on a continuum of closed to open (i.e., learning) genetic programs. A genetic program gives an individual preadaptation to behave in a certain manner. Therefore, preadaptation to certain environmental cues plays an important role in habitat selection, but the potential for learning may exist in some species.

Habitat preference

Habitat preference is the consequence of habitat selection, resulting in the disproportional use of some resources over others. Habitat preferences are most strikingly observed when animals spend a high proportion of time in habitats that are not very abundant on the landscape. Habitat availability Habitat availability is the accessibility and procurability of physical and biological components of a habitat by animals. Availability is in contrast to the abundance of resources, which refers only to their quantity in the habitat, irrespective of the organisms present. For example, the abundance of a prey species for a particular predator could be measured, yet not all of the prey in the habitat is available to the predator because there may be factors, (e.g., ample cover) that restrict their accessibility. Vegetation beyond the reach of an animal is not available as forage, even though the vegetation may be preferred. Measuring actual resource availability is important to understand wildlife habitat, but in practice it is seldom measured because of the difficulty of determining what is and what is not available. Consequently, quantification of availability usually consists of a priori or a posteriori measure of the abundance of resources in an area used by an animal, rather than true availability.

Habitat Is Species Specific

When I hear someone state “This is great wildlife habitat”, it is like walking into a brick wall and I can only guess what they mean. All the components necessary for reproduction and survival are not the same for all species and “great wildlife habitat” for one species may not even come close to serving as appropriate habitat for others. This has and will continue to be a problem because manipulations of the landscape will favour the habitats of some species but be detrimental to the habitats of others. A lot of effort has been placed on ecosystem management in the 1990s, but when considering specific organisms the manager needs to consider their unique array of requirements for survival. With a knowledge of habitat requirements for the species of interest, the manager can make informed decisions as to how landscape alterations will influence plant and animal communities.

Migration, navigation and orientation

Birds are not perfectly adapted to all environments and sometimes a bird has to leave – *migration*. In its purest sense, migration refers to seasonal movements between a location where an individual or population breeds and a location where it survives during the non-breeding period. Long-distance migrants are those that have a complete shift between the breeding and wintering areas, such as the Blackpoll Warbler that breeds in Canada; short-distance migrants, such as the Pine Warbler, make shorter trips, such as up and down the side of a mountain. Partial migrants are those species in which some individuals of a species leave and others stay, such as American Robins. Average distances covered are about 1000-3000 km each way, but 4000-6000 km is not uncommon.

A migratory journey is usually broken into a series of short flights. Most songbirds migrate at night, singly or in loose flocks, and my cover 300-600km per night but they don't fly every night and a 3000 km journey may take 3-4 weeks.

Birds might also have more than two migratory destinations. Anna's Hummingbird, for example, breeds in the coastal chaparral and valley of California, summers in the high mountains, and winters in the deserts of Arizona and Mexico. Birds tend to migrate along four major routes in North America called flyways, depicted below (Fig 1).

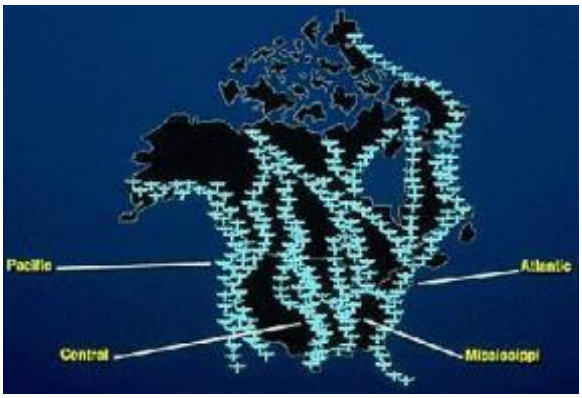

Fig 1: Migratory routes

Food Habits and Migration

There is often a relationship between food habits and migration. Insects become scarce in the winter in northern latitudes, so insectivorous birds must move closer to the equator where insects are more abundant. Insectivorous birds are the largest group of migrants. Some insect-eaters, such as chickadees, can eat seeds in the winter and thus do not tend to migrate. Fruit-eating birds may not have to migrate as far because fruits are generally available at the edge of the temperate zone. And seeds are available all year around in most areas, so seed-eaters tend not to migrate. But seeds are not produced in the winter and may be covered by snow, forcing birds southward, or at least down the mountains. By contrast, finches and crossbills that feed on pine seeds from the cones don't have that problem and are non-migratory.

Fish-eating birds may be cut off from their food supply when northern lakes freeze over, but can generally overwinter if the lake doesn't totally freeze over. Most aquatic birds winter at the edge of the temperate zone, but some go to the tropics. The most well-known of these is the Arctic Tern which migrates from the edges of the Arctic to the Antarctic and back again each year – a 22,000 mile round trip. Large hawks which feed on mammals and birds tend to migrate because their prey either hibernates or migrates. In addition, photoperiod shortens and they have less time for hunting. Owls,

on the other hand, don't migrate as much (as a group) because the shortening photoperiod is not a major factor to them.

Geography and Migratory Behaviour

There is more land area in the North Temperate Zone than there is in either the tropics or the South Temperate Zone; *i.e.* there is more potential breeding area than wintering area. There is even more contrast in habitat types; *e.g.* in the New World, the total amount of tropical forest in South and Central America is about equal to the total amount of forest in North America. But there is a limited amount of other habitat types in both areas. So a grassland species of North America must either winter in the south-western US or northern Mexico, or fly all the way to Venezuela to find grassland. Wetland species- for example, shorebirds that nest in the marshes of the Arctic tundra – have no comparable habitat in the south and thus winter along the coastlines or travel all the way to the grasslands of Argentina.

In the Old World, much of the northern land mass is forest or grassland, while most of Africa is desert and scrubland, only about 20% being tropical forest. Thus the birds that migrate have to adapt to living in a much different environment. But there are limits to this flexibility- a sandpiper can't live in a rainforest. Australia, being relatively isolated by contrast, has relatively few migratory species – only 8% show north-south migrations. But 26% are nomadic– that is, they travel around the continent continually. Although there is a large amount of tropical forest in South America, the vast majority of birds that migrate from North America to South America winter in Central America, or the West Indies – very few actually go to northern South America or into the interior of South America.

Site Fidelity

If a bird returns to the same site each year, it must have some sort of genetically-based ability to remember the site and how to get there. For example, a young warbler may carry genetic information that causes it to remember its place of birth and then respond to cues that guide it to Puerto Rico. The young bird then spends the winter moving around, searching for a good place to stay. After overwintering, it returns north to the area of its hatching. The next year it returns to the same site in Puerto Rico. So the bird may spend its life in only a few hectares in two sites thousands of miles apart. There must be a genetic basis to migration, because young birds that have never migrated before migrate successfully to their breeding grounds. In many shorebirds, the adults leave for their wintering grounds before the young, so the young have to know where to go without help.

Route Selection

Evolution of a migratory route is determined by many factors. Geographic or topographic factors are important for some species. But even some small warblers and hummingbirds have the ability to fly across the Atlantic Ocean on their way from North America to Mexico. But other species, such as some hawks and Turkey Vultures, follow the land route rather than crossing the ocean. Obviously, migratory routes have changed a great deal over geologic time; the glaciers of the ice ages advancing and retreating must have forced birds to change their routes, so migratory routes must have evolved like any other behaviour to changing conditions.

Timing of Migration

The timing of migration is controlled by both proximate and ultimate factors. Ultimate factors are evolutionary and proximate factors are cues.

Resource and Breeding Factors -ultimate factor that determines the suitability of the breeding and wintering grounds. Breeding sandpipers must arrive early in the year in the Arctic as soon as the snow clears so that they have enough food and sufficient time to raise their young before fall.

Climatic Factors – ultimate factor that determines food supply and survivability on the breeding or wintering grounds, but also a proximate factor that influences the timing of migration. Bad weather, rain, and heavy winds may cause birds to fly faster or slow them down. In the Northern Hemisphere, high pressure systems have winds that blow clockwise around them and low pressure systems have counter clockwise winds. So if there is a high pressure system (causing winds to blow from the north), followed by a low pressure system (causing winds to blow from the south), the birds will wait until the low pressure system starts to blow and get a tail wind on their way north. That's why there are often "waves" of migrants. Birds take about 24 hours to cross the Yucatan Peninsula to the US nonstop; with a tail wind this can be reduced to 20 hours. If the wind should switch direction, it could slow the birds down enough that they die enroute.

Photoperiod Clues – this is a proximal cue that tells the birds when to migrate. If the birds depended merely on the weather, they could leave too early and be caught with bad weather later or no food in their breeding site; if the weather is bad and they left too late, they would be outcompeted. So they depend on the photoperiod for timing as photoperiod is very predictable and, on the average, is most beneficial for migration.

Navigation and Orientation

How does a bird find its way? We can learn some things about the navigation and orientation behaviour of birds through observation or through banding, but the best information has come through experiments.

Species vary in their navigational abilities. Some birds have the ability to find their way back if they are displaced. Homing pigeons are pretty good at this and the most impressive was a Manx Shearwater, taken from its burrow on the Atlantic coast and flown to England by commercial aircraft, returned to the burrow in 12.5 days.

Most early studies on navigation tried to explain it by one mechanism. But now we know that birds use several mechanisms.

Landmarks. There is some indication that birds use landmarks, at least sometimes. Homing pigeons taken 200 miles off the coast of France in the middle of the Atlantic – only 2% returned home. But in other studies, pigeons were fitted with nearly opaque contact lenses – so they could could see light, but nothing else, and most found their way home (but had a hard time landing). And, of course, birds that navigate at night cannot see landmarks.

Solar Cues. The position of the sun seems to be one of the prime navigational cues for both nocturnal and diurnal fliers.

In the 1950s, Kramer put Starlings in cages with opaque walls and a glass top. He found that on sunny days, they oriented in the appropriate direction, but on cloudy days they oriented randomly. Bellerose, working with Blue-winged Teal, fitted them with radio transmitters and found that on cloudy days, they would circle until they got above the clouds at which time they started to move in the appropriate direction.

Using the sun as a compass requires compensating for its movement across the sky, which birds seem to be able to do by setting their internal biological clocks. One experiment with homing pigeons kept pigeons under artificial light until their biological clock was six hours off from the actual local time.

When released, they flew 90 degrees off from the proper direction. This internal clock is called a "sun compass".

Radio- tagged Blue-winged Teal were noted to circle under a cloudy sky until they got above the clouds and then headed in the proper direction, indicating that they need to see the sun to navigate. Even nocturnal birds seem to take their cue from the sun as cloudy days either result it no migration at night or the migrants show an unusual variation of direction.

Stellar Cues. Cues provided by the stars are obviously important only to nocturnal migrants. To determine the importance of stellar cues in orientation, birds were put in cages outside with a view of the night sky to see which way they oriented.

But the most sophisticated experiments put these birds not under the normal night sky, but in a planetarium. This way, the sky could be changed and the birds' response to the moving sky could be measured. In a classic experiment by a German ornithologist, Sauer, in the 1950's, warblers were put into cages in a planetarium in the fall. The birds oriented in a certain direction and Sauer moved the night sky in that direction (at a normal speed) about 300 Km, the distance the birds would normally move in a night. The next night he did the same thing – move the sky in the direction the birds were oriented. At the end of a couple of weeks, the birds quit showing their restlessness as they had migrated to the night sky that showed middle Africa, where they would have gone if they were free. Not only that, they migrated around the Mediterranean sea enroute. All by stars.

Geomagnetic Cues. Geomagnetism is perhaps the most controversial and least understood of the major possible navigational cues. Early studies by Merkel and Wiltschko (1964) placed birds in a large cement cage with no environmental clues at all; the birds oriented properly. But when they were put in a large steel cage, which would obviously affect magnetic lines of force around the cage, the birds oriented randomly.

The best studies have been done by Keeton in the 1970's with homing pigeons. When brass bars were attached to pigeons, they generally oriented themselves in the direction of home from an unfamiliar location, whether it was a sunny or overcast day. When a magnetic bar was attached to the pigeons, they did about the same on a sunny day, but were disoriented on an overcast day – the magnet seemed to have thrown them off when they had no sun to orient by. A more sophisticated experiment was done later with Helmholtz coils. The coils produced a magnetic current around the birds' heads. The current could either flow in a clockwise direction (normal direction of the magnetic field in the northern hemisphere), in which case the birds flew directly home on either a cloudy or sunny day; or the current could be made to go counter clockwise- in this case the birds flew directly home on a sunny day, but flew 180 degrees in the wrong direction on an overcast day. These experiments demonstrate two things: that geomagnetic lines of force do seem to be used for orientation and that the sun compass is the dominant navigation mechanism.

Thus orientation and navigation may be due to a variety of cues. There is some evidence that odours may play a role in navigation, or even sounds (ocean waves, waterfalls).

Probable questions:

1. Discuss the basic idea about memory.
2. Discuss short term memory.
3. What do you mean by ling term memory?
4. What do you mean by learning?
5. Discuss different types of learning.
6. Discuss different types of play.
7. What is formal and non-formal learning?
8. Name the factors affecting learning/ Give a detailed discussion about the factors.
9. How can you establish that habitat is species specific?
10. Discuss in details about the migratory behaviour.
11. Discuss the factors which controls the timing of migration.
12. What are the mechanisms by which bird can control its navigation?

Suggested Readings:

1. Alcock, J. (2001). Animal Behaviour: An Evolutionary Approach. , Sinauer Associate Inc., USA.

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5. Mathur, R. (2005). Animal Behaviour. Rastogi Pub. Meerut.

6. Refinetti, R. (2000). Circadian Physiology. CRC Press, Boca Raton.

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Unit-III

Kinship: Relatedness, inclusive fitness, Selfishness, altruism

Objective: In this unit you will know about kinship, relatedness with inclusive fitness, selfishness and altruism.

Introduction

Kinship is one of the main organizing principles of society. It is one of the basic social institutions found in every society. This institution establishes relationships between individuals and groups. People in all societies are bound together by various kinds of bonds.

The most basic bonds are those based on marriage and reproduction. Kinship refers to these bonds, and all other relationships resulting from them. Thus, the institution of kinship refers to a set of relationships and relatives formed thereof, based on blood relationships (consanguineal), or marriage (affinal).

Definition

'The social relationships deriving from blood ties (real and supposed) and marriage are collectively referred to as kinship.' – Abercrombie et al.

Kinship: The degree of coefficient of relationship (genetic or hereditary relatedness) between members of a species is called kinship.

Kin: The relatives of same species. Or kin is a group of individuals of common ancestry

Types of Kinship:

Kinship is of two types:

a. Affinal Kinship, and
b. Consanguineous Kinship.

Affinal Kinship:

The bond of marriage is called affinal kinship. When a person marries, he establishes relationship not only with the girl whom he marries but also with a number of other people in the girl's family. Moreover, it is not only the person marrying who gets bound to the family members of the girl but his family members also get bound to the family members of the girl.

Thus, a host of relations are created as soon as a marriage takes place. For example, after marriage a person becomes not only a husband, but he also becomes brother-in-law and son-in- law. Here it may be noted that in English language a number of relations created by marriage are referred by the same term. Thus, the same term 'brother-in-law is used for bahnoi, sala, jija and sadahu. On marriage a person also becomes foofa, nandoi and mausa.

Likewise a girl on marriage becomes not only a wife but also becomes daughter-in-law, she also becomes chachi, bhabhi, devrani, jethani, mami etc. Thus, marriage creates a host of relationships which are called affinal kin.

Consanguineous Kinship:

The bond of blood is called consanguineous kinship. The consanguineous kin are related through blood whereas the affinal kin are related through marriage. The bond between parents and their children and that between siblings is consanguineous kinship. Siblings are the children of the same parents.

Thus, son, brother, sister, uncle (chacha), elder uncle (taoo), nephew and cousin are consanguineous kin. i.e., related through blood. In this connection it may be pointed out that blood relationship may be actual as well as supposed.

Among polyandrous tribes the actual father of a child is unknown. An adopted child is treated as if it were one's own biologically produced child. Thus, blood relationship may be established not only on biological basis but also on the basis of social recognition.

Kinship and its degree

The relationship among individuals or people depends on the level of closeness and separation of its relationship. Closeness and distance are based on how these individuals are related to each other.

- **Primary kinship**

Primary kinship is based on direct relations. Individuals or people that are directly related are said to be primary in nature. Primary kinship is further divided into two:

- ***Primary consanguineal kinship*:** this kin refers to that kin that is directly related to each other by birth. For instance association with or amongst parents and children and among siblings.
- ***Primary Affinal kinship*:** the relation that takes place with marriage is said to be Primary Affinal kinship. The direct primary affinal kinship is the husband-wife relationship

- **Secondary kinship**

Secondary kinship alludes to the primary kinship. As it were, the individuals who are specifically identified with primary kinship (i.e. primary kin of our primary kinship) become secondary kinship. In other words, it means relations that come through primary kinship are said to be secondary kinship.

There are two types of Secondary kinship:

- ***Secondary consanguineal kinship**:* This kind of kin refers to primary consanguineal kinship. The basic example of secondary consanguineal kinship would be the relationship between grandparents and grandchildren.
- ***Secondary Affinal kinship:*** This kind of kinship refers to primary affinal kinship primary kinship. For example, Anita's husband is her primary affinal kinship and for Anita's husband, her parents and siblings are his primary kin. Therefore meaning the relationship between Anita and her sister in law/ brother in law or parents in law and more vice versa is said to be Secondary Affinal kinship. Also, your sibling's spouse and his/her parents in law will be his secondary affinal kinship.

- **Tertiary kinship**

Tertiary kinship is the secondary kinship of our primary kin or primary kin of our secondary kinship. For example, wife of our brother in law would be related to us as tertiary kin.

Tertiary kinship is further divided into two:

- ***Tertiary consanguineal kinship:*** An example of tertiary consanguineal kin would be our primary consanguineal kins (i.e. our parents) primary kins (i.e. our parents' parents meaning our grandparents) primary kins. (i.e. our grandparents' parents).
- ***Tertiary Affinal kinship:*** It means primary affinal kins primary kin or secondary affinal primary kin or primary affinal kins secondary kin. For example, our spouse's grandparents or grand uncles and aunties.

Importance of Kinship

Kinship has several importance in a social structure.

1. Kinship decides who can marry with whom and where marital relationships are taboo.
2. It determines the rights and obligations of the members in all the sacraments and religious practices from birth to death in family life.
3. Importance of kinship is observed on the occasion of marriage and family functions.
4. It determines family line relationships i.e. gotra and kula.

Inclusive fitness

Basic idea

Cooperation is abundant throughout the natural world and exists at all biological levels, from genes forming genomes to individuals collaborating in societies. Nature documentaries are frequently packed with stunning examples, from kamikaze bees stinging intruders to save the lives of their nest mates to meerkat helpers feeding the pups of others. However, beneath this appearance of kindness lies one of the most challenging issues for evolutionary theory. The problem is that natural selection favors genes that increase an organism's ability to survive and reproduce and so how can behavior that benefits others ever evolve? To simplify this problem the complex spectrum of social behaviors can be broken down into pair-wise interactions and classified according to the direct fitness benefits (number of offspring an individual produces stripped of social interactions) and costs to the actors and recipients involved. This leads to four types of behavior: Selfishness (benefit to actor, cost to the recipient) and mutually beneficial interactions (benefit to actor, benefit to the recipient) are easily understood as they increase the direct fitness of the actor. Altruism (cost to the actor, benefit to the recipient) and spite (cost to the actor, cost to the recipient), on the other hand, present an evolutionary paradox—how can a gene that is disadvantageous to an individual spread in a population? Darwin realized this problem, but it wasn't until 1963–1964, when William (Bill) D. Hamilton produced his benchmark papers, that it became clear how actions that decrease direct fitness can evolve through natural selection. Hamilton coined the term **"Inclusive Fitness"** to emphasize that the quantity that individuals attempt to maximize is not simply direct fitness, but also something called indirect fitness—the effect individuals have on the number of offspring everybody else in the population produces weighted by their relatedness. Inclusive fitness theory remains one of the most active areas of evolutionary research and provides an extremely important tool for understanding both the process and purpose of evolution.

Biology

Inclusive fitness**,** theory in evolutionary biology in which an organism's genetic success is believed to be derived from cooperation and altruistic behaviour. Inclusive fitness theory suggests that altruism among organisms who share a given percentage of genes enables those genes to be

passed on to subsequent generations. In this way, an altruistic act that supports the survival of a relative or other individual theoretically enhances the genetic fitness of both the recipient of the act and the altruistic organism. The propagation of shared genes was believed to be an underlying mechanism for the evolution of eusociality (cooperative behaviour characterized by division of labour and group integration that is found in certain species of animals, mainly social insects).

The idea of inclusive fitness was first proposed in 1932 by British geneticist J.B.S. Haldane in *The Causes of Evolution*. The theory was later named and developed by British evolutionary biologist William Donald Hamilton, who used inclusive fitness to explain direct (reproductive) and indirect (aided by a relative or a colony member) inheritance of genetic traits associated with altruism. Hamilton presented his inclusive fitness theory in 1963; the following year British evolutionary biologist John Maynard Smith coined the term *kin selection* to describe Hamilton's theory. Inclusive fitness later came to be understood as forming a general basis for kin selection theory, which attempts to interpret altruistic social behaviour in animals through genetic relatedness and benefits and costs associated with altruistic acts. Thus, in contrast to inclusive fitness, which considers genetic traits in both related and unrelated individuals, kin selection is concerned only with relatives. Hamilton's inclusive fitness theory, as well as kin selection, seemed too many biologists to reconcile the conflict between natural selection, in which "selfish" genes perpetuate their own fitness through survival of the fittest, and selfless behaviour, in which eusocial genes shared by relatives and colony members influence cooperative behaviour that encourage the propagation of those genes.

Inclusive fitness theory is most commonly applied to eusocial organisms, such as bees and ants, although it has also been invoked to explain cooperative breeding in animals such as birds and the adoption of orphaned young by asocial red squirrels (*Tamiasciurus hudsonicus*). In certain bird species, such as the Florida scrub jay (*Aphelocoma coerulescens*) and the groove-billed ani (*Crotophaga sulcirostris*), some individuals will stay near nesting sites and participate in the rearing of related offspring. Individuals that do not disperse to their own territories have been thought to perceive the inclusive fitness gains of cooperative breeding as being greater than fitness gains offered by dispersal to potentially less-favourable territory. In such instances, inclusive fitness through cooperative breeding is the result of constraints on territory quality and is influenced by factors such as food, mate attraction, and predation. Indeed, in the absence of constraints, staying near relatives is less advantageous, potentially limiting breeding opportunities and thereby making kin selection and inclusive fitness less beneficial to reproductive success. The amount of labour that cooperative breeding individuals contribute to raising relatives is variable. In contrast, eusocial organisms have fixed and stereotyped divisions of labour; castes such as sterile workers presumably accumulate reproductive advantages by helping their relatives in the cooperative raising of young.

Hamilton's rule

The potential genetic return depends on the relatedness between two individuals. Therefore, closer relationship gives greater potential genetic return.

It is expressed as-

$B/C > 1/r$ or $rB > C$ or $rB-C > 0$

Where, B= Benefits of altruistic act

C= Costs of altruistic act

r= Coefficient of relationship

Example: An altruist cannot produce one offspring of its own but its altruistic act saves five (5) nephews from perishing (accident). The value of r between non related is 0, between uncle & nephews is 0.25 and between parent and offspring is 0.5. Is this act net gain for altruist?

Ans: According to Hamilton's rule, we know that-

B/C > 1/r

Here, B = 5 x 0.25 =1.25 & C = 1 x 0.25 = 0.25

B/C = 1.25 / 0.25 = 5

And, 1/r = 1/0.25 = 4

So, B/C > 1/r

Therefore, Altruistic act will make net gain for the altruist

Kin Selection and Inclusive Fitness

The basic idea of kin selection is simple. Imagine a gene which causes its bearer to behave altruistically towards other organisms, e.g. by sharing food with them. Organisms without the gene are selfish—they keep all their food for themselves, and sometimes get handouts from the altruists. Clearly the altruists will be at a fitness disadvantage, so we should expect the altruistic gene to be eliminated from the population. However, suppose that altruists are discriminating in who they share food with. They do not share with just anybody, but only with their relatives. This immediately changes things. For relatives are genetically similar—they share genes with one another. So when an organism carrying the altruistic gene shares his food, there is a certain probability that the recipients of the food will also carry copies of that gene. (How probable depends on how closely related they are.) This means that the altruistic gene can in principle spread by natural selection. The gene causes an organism to behave in a way which reduces its own fitness but boosts the fitness of its relatives—who have a greater than average chance of carrying the gene themselves. So the overall effect of the behaviour may be to increase the number of copies of the altruistic gene found in the next generation, and thus the incidence of the altruistic behaviour itself.

Though this argument was first made explicit by William Hamilton (1964). Hamilton demonstrated rigorously that an altruistic gene will be favoured by natural selection when a certain condition, known as *Hamilton's rule*, is satisfied. In its simplest version, the rule states that $b > c/r$, where c is the cost incurred by the altruist (the donor), b is the benefit received by the recipients of the altruism, and r is the *co-efficient of relationship* between donor and recipient. The costs and benefits are measured in terms of reproductive fitness. The co-efficient of relationship depends on the genealogical relation between donor and recipient—it is defined as the probability that donor and recipient share genes at a given locus that are 'identical by descent'. (Two genes are identical by descent if they are copies of a single gene in a shared ancestor.)

Though Hamilton himself did not use the term, his idea quickly became known as 'kin selection', for obvious reasons. Kin selection theory predicts that animals are more likely to behave altruistically towards their relatives than towards unrelated members of their species. Moreover, it predicts that the *degree* of altruism will be greater, the closer the relationship. In the years since Hamilton's theory was devised, these predictions have been amply confirmed by empirical work. For example, in various bird species, it has been found that 'helper' birds are much more likely to help relatives raise their young, than they are to help unrelated breeding pairs. Similarly, studies of Japanese macaques have shown that altruistic actions, such as defending others from attack, tend to be preferentially

directed towards close kin. In most social insect species, a peculiarity of the genetic system known as ‘haplodiploidy’ means that females on average share more genes with their sisters than with their own offspring. So a female may well be able to get more genes into the next generation by helping the queen reproduce, hence increasing the number of sisters she will have, rather than by having offspring of her own. Kin selection theory therefore provides a neat explanation of how sterility in the social insects may have evolved by Darwinian means.

Kin selection theory is often presented as a triumph of the ‘gene's-eye view of evolution’, which sees organic evolution as the result of competition among genes for increased representation in the gene-pool, and individual organisms as mere ‘vehicles’ that genes have constructed to aid their propagation. The gene's eye-view is certainly the easiest way of understanding kin selection, and was employed by Hamilton himself in his 1964 papers. Altruism seems anomalous from the individual organism's point of view, but from the gene's point of view it makes good sense. A gene wants to maximize the number of copies of itself that are found in the next generation; one way of doing that is to cause its host organism to behave altruistically towards other bearers of the gene, so long as the costs and benefits satisfy the Hamilton inequality. But interestingly, Hamilton showed that kin selection can also be understood from the organism's point of view. Though an altruistic behaviour which spreads by kin selection reduces the organism's personal fitness (by definition), it increases what Hamilton called the organism's *inclusive* fitness. An organism's inclusive fitness is defined as its personal fitness, plus the sum of its weighted effects on the fitness of every other organism in the population, the weights determined by the coefficient of relationship r. Given this definition, natural selection will act to maximize the inclusive fitness of individuals in the population. Instead of thinking in terms of selfish genes trying to maximize their future representation in the gene-pool, we can think in terms of organisms trying to maximize their inclusive fitness. Most people find the ‘gene's eye’ approach to kin selection heuristically simpler than the inclusive fitness approach, but mathematically they are in fact equivalent.

Contrary to what is sometimes thought, kin selection does not require that animals must have the ability to discriminate relatives from non-relatives, less still to calculate coefficients of relationship. Many animals can in fact recognize their kin, often by smell, but kin selection can operate in the absence of such an ability. Hamilton's inequality can be satisfied so long as an animal behaves altruistically towards others animals that are *in fact* its relatives. The animal *might* achieve this by having the ability to tell relatives from non-relatives, but this is not the only possibility. An alternative is to use some proximal indicator of kinship. For example, if an animal behaves altruistically towards those in its immediate vicinity, then the recipients of the altruism are likely to be relatives, given that relatives tend to live near each other. No ability to recognize kin is presupposed. Cuckoos exploit precisely this fact, free-riding on the innate tendency of birds to care for the young in their nests.

Another popular misconception is that kin selection theory is committed to ‘genetic determinism’, the idea that genes rigidly determine or control behaviour. Though some sociobiologists have made incautious remarks to this effect, evolutionary theories of behaviour, including kin selection, are not committed to it. So long as the behaviours in question have a genetical *component,* i.e. are influenced to some extent by one or more genetic factor, then the theories can apply. When Hamilton (1964) talks about a gene which ‘causes’ altruism, this is really shorthand for a gene which increases the probability that its bearer will behave altruistically, to some degree. This is much weaker than saying that the behaviour is genetically ‘determined’, and is quite compatible with the existence of strong environmental influences on the behaviour's expression. Kin selection theory does not deny the truism that all traits are affected by both genes and environment. Nor does it deny that many interesting animal behaviours are transmitted through non-genetical means, such as imitation and social learning.

The importance of kinship for the evolution of altruism is very widely accepted today, on both theoretical and empirical grounds. However, kinship is really only a way of ensuring that altruists and recipients both carry copies of the altruistic gene, which is the fundamental requirement. If altruism is to evolve, it must be the case that the recipients of altruistic actions have a greater than average probability of being altruists themselves. Kin-directed altruism is the most obvious way of satisfying this condition, but there are other possibilities too. For example, if the gene that causes altruism also causes animals to favour a particular feeding ground (for whatever reason), then the required correlation between donor and recipient may be generated. It is this correlation, however brought about, that is necessary for altruism to evolve. This point was noted by Hamilton himself in the 1970s: he stressed that the coefficient of relationship of his 1964 papers should really be replaced with a more general correlation coefficient, which reflects the probability that altruist and recipient share genes, whether because of kinship or not. This point is theoretically important, and has not always been recognized; but in practice, kinship remains the most important source of statistical associations between altruists and recipients.

Altruism

What is Altruism?

You're hanging out with your friends while studying one day, and you open up a bag of chips. Being the good-natured person that you are, you offer to share your chips with your friends, even though it means there's less available for you. Your friends compliment you and thank you for your altruistic behavior. In the human world, altruism is an act of selflessness, where an action is taken that doesn't benefit the person doing it.

The same concept applies to biology. In biology, altruism means any individual acting in a way that reduces their own fitness while increasing the fitness of the group. Today we're going to learn why that happens and which groups of animals have taken their relationships to the next level.

Examples of Altruism

Although we as humans are used to taking care of each other, not many animals do this. Most are in direct competition with each other outside of their offspring. Not all animals believe sharing is caring! However, some animals that live in more complex social organizations got the memo.

Fig: Meerkats on high alert

For example, meerkats, which are found in Africa, are small carnivorous mammals that live underground. Meerkats stand tall on the ground, and when a predator such as a hawk approaches, one meerkat will call out an alarm. The alarm draws the hawk's attention to the watchman, while the other meerkats can escape safely into the burrow.

Vampire bats have been known to regurgitate blood to bats that are sick or were unable to find food, even though it means less food for themselves. Good thing you can just give the bag of chips to your friends, right?

Even complex societies of insects are known for their altruism. Termites and bee societies are made of sterile drones. They cannot reproduce, but only forage and build the nest for the queen. The worker insects have no purpose but to serve their colony in these ways.

Altruism and Natural Selection

Altruism is the opposite of what we normally think about in evolution, which is survival of the fittest. A famous scientist named Charles Darwin came up with the idea that all life is competing to survive and reproduce, called natural selection. Individuals that survive to pass on their genes are more fit than those that don't. Altruism rubs against this line of thinking.

In some communities, certain individuals will sacrifice their well-being for the overall good of the group. Essentially, they're taking a hit for the team. In the vain of natural selection, this makes no sense. If an altruistic organism is sacrificing itself for the group, how will its genes be passed on so that there are more altruistic individuals in the future?

Altruism and Kin selection

Altruism is a phenomenon in which one individual benefits the other at its own expense. The phenomenon occurs in social animals or in closely knit populations, and is considered a paradox of natural selection theory. How can a gene that benefits other individuals at the expense of the bearer be favoured by natural selection? But kin selection favours traits that result in decreased personal fitness provided they increase the survival and reproductive fitness of the species, population or family.

Kin selection works not on individuals but on genotypes. An altruist by way of helping other individuals increases the fitness of its own genome. A honey bee worker is a sterile female and shares at least 50% of its genotype with its sisters even when its mother and father are unrelated. Workers share only 25% genes with haploid brother drones. If a worker decides to breed on its own, its diploid daughters and haploid sons will never be more than 50% related to it. So, the worker becomes sterile and ensures the survival of her genetically identical sisters because then queen can produce more offspring than workers reproducing individually.

The equation shows that a gene that favours altruism could spread when participants are related and the cost to the actor is low as compared to the benefit to the recipient. Altruism always pays when $\boldsymbol{Br>C.}$ Therefore, altruism is promoted by kin selection and close genetic relationship.

In a large number of bird species, especially those in which nesting opportunities are limited, young ones help their parents in rearing their own sisters and brothers by way of nest building, nest defense and feeding the chicks, although they are themselves capable of breeding. In such birds, as for example in bee-eaters, help is always given to their kin, and the importance of this assistance can be gauged by the fact that there is considerable mortality of chicks due to starvation if such a help is withdrawn.

Reciprocal altruism and Group selection

The theory of group selection was championed by Wynne-Edwards (1962). Altruism has evolved among the related individuals by means of kin selection. But there are instances of cooperation among the unrelated individuals. Altruistic act towards non-kin is possible only if the recipient is likely to return the favour at a later date, in a *'Tit for Tat'* manner.

Natural selection will favour altruism among unrelated individuals only if they reciprocate, and then this is called Group selection, which will select out selfish individuals from the population. Robert Trivers (1971) proposed that reciprocal altruism can develop in the following conditions:

1. If interacting individuals remain together for considerably longer time.

2. If frequency of altruistic attempts is high.

3. If the cost and benefit to both individuals are more or less equal.

4. If individuals that fail to reciprocate are punished in some way, such as withdrawing the benefits in future.

Species which have mutual dependence in defence, foraging, territoriality etc. are

Most likely to develop reciprocal altruism, as in monkeys, baboons, chimpanzees and man. Kin selection and reciprocal altruism are sometimes found to coexist in many social groups of animals and at times it is difficult to distinguish between the two or measure them independently.

Selfishness

Selfishness is the tendency to prioritize one's own desires and needs above the needs and desires of other people.

What is Selfishness?

We are all born with a drive to stay alive and healthy, and selfishness may be a misplaced manifestation of this. A certain degree of selfishness is normal. For example, many people would choose to ensure their own food needs are met before giving food to others. But selfishness can also be a pathological personality trait. Selfish people may prioritize their own petty needs above the significant needs of others. For example, a person is exhibiting selfishness when he or she steals money from their mother to buy a comic book.

Some mental health problems can contribute to the development of selfishness. Many personality disorders, particularly antisocial personality disorder and narcissistic personality disorder, cause people to be so wrapped up in their own desires that they either do not notice or do not care about the needs of others. Many other mental illnesses can cause extreme self-involvement, which can contribute to selfishness. A depressed person, for example, might be so wrapped up in his or her own feelings of suffering that he/she is unable to provide for his/his children or communicate with his/her partner.

Different Conceptions of Selfishness

Many religions decry selfishness and emphasize the virtues of compassion, empathy, and self-sacrifice. The pacifist movement, which draws on many religious traditions, is a radical answer to selfishness, and emphasizes non-violence even in the face of overwhelming hostility. Some religious gurus have advocated extreme self-sacrifice, emphasizing the primacy of others over oneself.

There is significant debate in evolutionary biology about the evolved nature of selfishness. Richard Dawkins' book *The Selfish Gene*, for example, argues that our genes have the "selfish" desire to propagate themselves and do nothing else. Some biologists argue that people are innately selfish. Others, however, emphasize that helping others can ensure the survival of the species and argue that compassion, empathy, and self-sacrifice are as innate to people as selfishness. People are sometimes more likely to show self-sacrificing behaviour for close relatives, and some biologists argue that this is an evolved trait. Many parents would give up their own lives for the lives of their children; one interpretation of this inclination is that when a child survives, the parent's genes survive with the child.

Selfishness as second-order altruism

Altruism and selfishness, like free will and determinism, seem to be polar opposites. Selfishness is seldom considered a group-beneficial strategy. In the typical evolutionary formulation, altruism benefits the group, selfishness undermines altruism, and the purpose of the model is to identify mechanisms, such as kinship or reciprocity that enable altruism to evolve. Recent models have explored punishment as an important mechanism favouring the evolution of altruism, but punishment can be costly to the punisher, making it a form of second-order altruism. This model identifies a strategy called "selfish punisher" that involves behaving selfishly in first-order interactions and altruistically in second-order interactions by punishing other selfish individuals. Selfish punishers cause selfishness to be a self-limiting strategy, enabling altruists to coexist in a stable equilibrium. This polymorphism can be regarded as a division of labour, or mutualism, in which the benefits obtained by first-order selfishness help to "pay" for second-order altruism.

Selfishness is rarely described as a group-beneficial strategy. Selfish strategies are labelled as deviant, cheating, free-riding, egoistic, but most of all, as undermining altruism and cooperation. In contrast, altruistic and cooperative strategies, almost by definition, benefit the group, often at the expense of the individual actor. In the typical evolutionary model, the invasion of selfish strategies into a group leads to the dissolution of altruism. Examples include scroungers among foraging groups, infanticide of unrelated infants, sneaking worker reproduction in eusocial insect colonies, and failure to help in territorial defence.

Probable questions:

1. Define kinship, kin selection and kin.
2. What do you mean by Consanguineous Kinship?
3. What do you mean by Affinal Kinship?
4. Discuss the degree of kinship on the basis of closeness and distance.
5. Discuss secondary kinship.
6. Write down the importance of kinship.
7. Write short notes on inclusive fitness.
8. What is Hamilton's rule? Elaborate this rule with an example.
9. What do you mean by inclusive fitness?
10. Discuss the relationship between kin selection and inclusive fitness.
11. What do you mean by altruism? Give example.
12. What is reciprocal altruism? Give example.
13. Discuss the relationship between altruism and kin selection emphasising on natural selection.
14. What is selfishness?
15. Discuss the selfishness as second order altruism.

Suggested Readings:

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4. Mandal, F. (2010). A Text Book of Animal Behaviour. Pentice Hall India.

5. Mathur, R. (2005). Animal Behaviour. Rastogi Pub. Meerut.

6. Refinetti, R. (2000). Circadian Physiology. CRC Press, Boca Raton.

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Unit IV

Conflict: Sexual selection, aggression, competition dominance, Infanticide

Objective: In this unit you will know about conflict; Sexual selection, aggression, competition dominance and infanticide

Sexual Selection

Sexual selection is a mode of natural selection in which members of one biological sex choose mates of the other sex to mate with (intersexual selection), and compete with members of the same sex for access to members of the opposite sex (intrasexual selection). Sexual selection, theory in postulating that the evolution of certain conspicuous physical traits—such as pronounced coloration, increased size, or striking adornments—in animals may grant the possessors of these traits greater success in obtaining mates. From the perspective of natural selection, such increases in mating opportunities outweigh the risks associated with the animal's increased visibility in its environment. This concept was initially put forth by English naturalist Charles Darwin in The Descent of Man (1871).

Mutual attraction between the sexes is an important factor in reproduction. Particularly in birds and mammals, the males are often larger and stronger, more brightly coloured, or endowed with conspicuous ornamentation. These traits, however, make animals more visible to predators—the long plumage of male peacocks (*Pavo cristatus*) and birds of paradise (*Paradisaea*) and the enormous antlers of aged male deer (*Odocoileus*) (Fig 1) are cumbersome loads in the best of cases. Darwin knew that natural selection could not be expected to favour the evolution of disadvantageous traits, and he was able to offer a solution to this problem. He proposed that such traits arise by "sexual selection," which "depends not on a struggle for existence in relation to other organic beings or to external conditions but on a struggle between the individuals of one sex, generally the males, for the possession of the other sex."

The presence of a particular trait among the members of one sex can make them somehow more attractive to the opposite sex. This type of "sex appeal" has been experimentally demonstrated in all sorts of animals, from vinegar flies (*Drosophila*) to pigeons, mice, dogs (*Canis familiaris*), and rhesus monkeys (*Macacca mulatta*). When, for example, *Drosophila* flies, some with yellow bodies as a result of spontaneous mutation and others with the normal yellowish grey pigmentation, are placed together, normal males are preferred over yellow males by females with either body colour.

Fig 1: A pair of red deer stags (*Cervus elaphus*) competing for possession of a female in the rutting season.

Intersexual Selection and Intrasexual Selection

Before we delve into the types of sexual selection, it's worth taking a moment to differentiate between inter and intra. *Inter* means between groups. For example an *inter*state highway goes between the states. Or the *Inter*net is a network that goes between computers. Contrast this with *intra*, which means within groups. An intrastate highway is a highway that travels inside a state's boundaries. Or intramural sports are sports that are played within your college, but not against other colleges.

Okay, so let's start with **intersexual selection**, which is sexual selection that is *between* two sexes. For example, consider a male peacock (Fig 2). Their giant, brightly coloured tails make avoiding predators difficult, so what's the advantage of having such tails? Well, females choose male peacocks with brightly coloured tails, so even though it's risky to be parading around predators with a bright tail, your chances of mating (and reproducing) are increased and thus this trait gets passed down. The peacock is an example of sexual selection *between* the two sexes, or intersexual selection. Just like a muscular, bearded human, it's assumed that female peacocks choose brightly coloured male peacocks because in order to produce such a large, colourful tail the male peacock must have good genes. These good genes, in turn, will contribute to the success of the offspring.

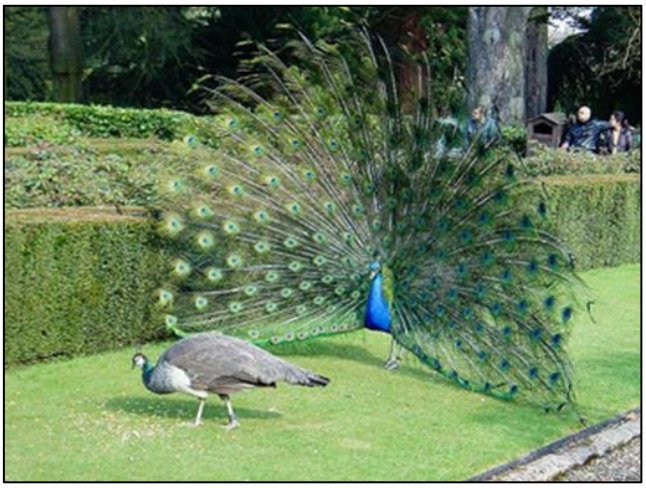

Fig 2: Female peacocks choosing males with brightly coloured, large tails is an example of Intersexual Selection

Contrast this with **intrasexual selection**, which is sexual selection *within* a sex. An example here is the competition seen in male primates. In many primates, a male will attempt to keep other males away from females so he can be their primary mate. Large, aggressive male primates with large canine teeth are usually the ones that are able to mate and pass along their genetic material. So here it is not selection from a different sex, its selection within the same sex. Again, this ensures the female gets the best genetic material from the male because in order for a male to grow large canines and defeat other males, he must be healthy and carry good genetic material.

How Does Sexual Selection Operate?

Sexual selection can operate both intra- and inter-sexually, either sequentially or simultaneously. During intrasexual selection, members of the same sex attempt to outcompete rivals, often during direct encounters. Intrasexual selection is typically responsible for the evolution of male armaments such as deer antlers, beetle horns, and large body size, which provide individuals with an advantage when fighting off potential competitors. Individuals who are better able to exclude competitors, have a greater chance to acquire mates and father offspring. For example, dominant male red deer

monopolize a group of females (also known as harem) by constantly fighting off competitors, and they father most of the offspring produced by the females. By contrast, intersexual selection results from interactions between the sexes, typically involving mate choice. The evolution of elaborate behavioural displays and morphological traits can often be explained as the result of intersexual selection. Usually, females tend to be choosier, evaluating morphological and behavioural traits from potential mates to determine which will maximize their fitness. Males tend to compete with one another to gain the female's attention. An extreme example of intersexual selection can be found in species where males form leks where multiple males gather to display to females.

Bateman's principle

Bateman's principle, in evolutionary biology, is that in most species, variability in reproductive success (or reproductive variance) is greater in males than in females. It was first proposed by Angus John Bateman (1919–1996), an English geneticist. Bateman suggested that,

i. Since males are capable of producing millions of sperm cells with little effort, while females invest much higher levels of energy in order to nurture a relatively small number of eggs, the female plays a significantly larger role in their offspring's reproductive success.

ii. Females should be the choosier sex because eggs are expensive to produce and because a female's potential reproductive success is limited compared with that of a male.

iii. Female's greater choosiness in mate selection should translate into greater variance in the reproductive success of males (Bateman, 1948).

Bateman's paradigm thus views females as the limiting factor of parental investment, over which males will compete in order to copulate successfully.

Darwin argued that, all else being equal, any male trait that confers mating and fertilization advantages and is passed down across generations will, over time, increase in frequency in a population, because males with such traits will produce more offspring than their competitors. Darwin's idea about the struggle among males for mating opportunities forms one basic foundation of our current understanding of sexual selection. Of course, from a proximate perspective, the ways in which hormones, neurobiology, development, environment, and many other factors operate on any particular behaviour play a very important role in the sexual selection process as well.

Competition for mates can take many forms, depending on ecology, demography, and cognitive ability. For example, males may fight among themselves, occasionally in dramatic "battles to the death," but often in less dangerous bouts, to gain mating opportunities with females. This latter form of male-male sexual competition is illustrated by male stag beetles and red deer (*Cervus elaphus*), which use their "horns" (enlarged jaws) and antlers, respectively, in physical fights over females; the winners of such contests mate more often than the losers.

Aggression

Introduction

- In psychology, the term aggression refers to a range of behaviours that can result in both physical and psychological harm to yourself, others, or objects in the environment. This type of behaviour centres on harming another person either physically or mentally. It can be a sign of an underlying mental health disorder, a substance use disorder, or a medical disorder. Predatory behaviour between members of one species towards another species is also described as aggression.
- To exhibit aggression towards members of another species is common.

Examples: Lions are aggressive hunters of antelopes; Eagles are aggressive hunters of small mammals.

- Aggression is defined as an agonistic behaviour which is a system of behaviour pattern that has the common function of adjustment in situations of conflict among conspecifics.
- The term agonistic includes all aspects of conflicts such as threat, submission, chases and physical combat.
- Most agonistic behaviour involves competition for some resources. Like most behaviours, aggression can be examined in terms of its ability to help an animal reproduce and survive.
- Animals may use aggression to gain and secure territories, as well as other resources including food, water, and mating opportunities. Researchers have theorized that aggression and the capacity for murder are products of our evolutionary past.

The Nature of Animal Aggression

Aggression sometimes occurs when parents defend their young from attack by members of their own species. Female mice, for example, defend their pups against hostile neighbours, while male stickleback fish defend eggs and fry against cannibalistic attack. More frequently, however, animals fight over resources such as food and shelter—e.g., vultures fight over access to carcasses, and hermit crabs fight over empty shells. Another important resource over which fighting commonly occurs is potential mates. In this case the biology of gamete production has an influence on aggressive behaviour because a female's eggs are larger, are fewer in number, and require more energy to produce than a male's sperm. Competition among males over females is usually more frequent and intense than competition among females over males. As a result, the most spectacular fights among animals, whether they are crickets, salmon, tree frogs, chaffinches, or stags, occur between males over fertile females.

Aggression may be focused on a specific area, such as a defended territory from which rivals are vigorously excluded. A notable example is shown by mudskippers. Intertidal fish that defend small territories where they browse on microscopic plants. The fish build mud walls around the borders of their territories, and at low tide water is retained within the walls (incidentally permitting the human observer to visualize the mosaic of territories in a colony of these fish). Territorial behaviour is also shown by rag worms and fiddler crabs when they defend their burrows, by male dragonflies and sticklebacks defending breeding grounds, by male tree frogs, sage grouse, and Uganda kob defending high-quality sites for courting and mating, and by spiders, reef fish, and hyenas when they defend feeding areas.

A common feature of aggression in most species is that fights tend to start with relatively harmless displays or postures. For example, aggressive interactions between two red deer stags begin with an exchange of deep roars followed by a display of "parallel walking," in which the stags strut side by side assessing their relative size. The aggression may then escalate to direct attacks during which the stags charge at each other, stabbing and wrestling with their antlers. Most confrontations are resolved early while displaying, but many others continue to the point of intense and dangerous fighting.

Contrary to previous assumptions, injury and death during animal fights are not uncommon. In species where animals live in established groups, however, overt fighting is often replaced by a set of relationships in which a subordinate individual consistently defers to a dominant one. Wolf packs, for example, are known for their clear hierarchical relationships. When two group members meet, the dominant animal adopts an upright stance, with raised ears and tail, while the subordinate flattens its body to the ground with the ears against the head and the tail lowered, a submissive posture that serves to protect it from attack. In a number of bird species, variations in plumage act as "badges of

status," especially in large winter flocks. The black throat patch or bib of the house sparrow and the dark chest stripe of the great tit are signals of status; dominant individuals have more-conspicuous bibs or stripes than do subordinates and thus have preferential access to food.

Types of Aggression

Psychologists distinguish between two different types of aggression:

- **Impulsive Aggression:** Also known as affective aggression, impulsive aggression is characterized by strong emotions, usually anger. This form of aggression is not planned and often takes place in the heat of the moment. When another car cuts you off in traffic and you begin yelling and berating the other driver, you're experiencing impulsive aggression. Research suggests that impulsive aggression, especially when it's caused by anger, triggers the acute threat response system in the brain, involving the amygdala, hypothalamus, and periaqueductal grey (PAG).
- **Instrumental Aggression:** Also known as predatory aggression, instrumental aggression is marked by behaviours that are intended to achieve a larger goal. Instrumental aggression is often carefully planned and usually exists as a means to an end. Hurting another person in a robbery or car-jacking is an example of this type of aggression. The aggressor's goal is to obtain money or a vehicle, and harming another individual is the means to achieve that aim.

Other Forms of Aggression

- *Territorial* – Exclusion of others from a physical space to maintain its individual distance. E.g. New Zealand gannets.
- *Dominance* – Animals remember each other by previous encounters and maintain their status as dominant or submissive. E.g. dogs, monkeys, wolves and birds. Dominance may be linear, triangular and Coalition.
- *Sexual* – Use of threat and physical punishment, usually by males to obtain and retain females called as combat.
- *Parent-offspring* – Disciplinary action against off-springs.
- *Weaning* – Restriction of access of off-springs to milk.
- *Predatory* – Act of predation, possibly includes cannibalism. This is called as aberrant behaviour.
- *Behavioural* - Behaviour between members of the same species shows different aggression such as- violent, resolve conflicts, establish social order, warnings, defence of resources, instil fear in others, communication and defend offspring

Purposes of Aggression

Aggression can serve a number of different purposes, including:

- To express anger or hostility
- To assert dominance
- To intimidate or threaten
- To achieve a goal
- To express possession
- A response to fear
- A reaction to pain
- To compete with others

Factors affecting aggression

Physiological Causes of Aggression

The immediate cause or motivation of an attack by one animal on another lies in the attacker's response to certain cues or stimuli. Such cues can be visual (robins will vigorously attack a bunch of red feathers placed in their territory), auditory (robins will also attack a tape recorder playing the song of another robin), tactile (spiders respond to vibrations set up by rivals entering their web), olfactory (the scent of urine from another male mouse elicits vigorous attack from a territorial male), and even electrical (to deter territorial intruders, gymnotid and mormyrid fish use electrical signals generated by modified muscles). Often full attack is elicited by a combination of such cues. And yet aggression is not an inflexible response inevitably triggered by a particular stimulus or by collections of stimuli. Depending on the internal state of the potential attacker, the same opponent may be attacked on one occasion but ignored on another. In particular, an individual's tendency to attack a rival is influenced by the activity of key structures and pathways in the nervous system and by the levels of particular hormones circulating in the blood.

Neuroendocrine influences

The neuroendocrine mechanisms that generate aggressive responses and modulate the levels of aggression are complex and far from fully understood. They have been best-documented in invertebrates, particularly in lobsters and crayfish, where the neural circuits responsible for the performance of displays during fights have been partially identified. These crustaceans fight readily, and, after a series of interactions between the same individuals, a hierarchical relationship is established whereby the victor consistently takes a dominant posture, with raised legs and forward-directed antennae, while the loser adopts a submissive posture and avoids future fights. The neurohormone serotonin is clearly involved in the control of aggression and dominance, as is octopamine (an invertebrate analog of norepinephrine, or noradrenaline, which in vertebrates acts in response to stressful situations). Serotonin injections cause lobsters to take up the dominant posture, while octopamine injections induce submissive postures. In addition, when the levels of serotonin in subordinate animals are experimentally increased, the willingness of the animals to fight also increases. At least two pairs of serotonin-containing nerve cells have been identified in the central nervous system (CNS). These have connections with the motor neurons responsible for generating dominant and subordinate postures and with the motor neurons promoting more intense attack and escape. How the system is activated varies depending on the social status of the animal concerned; activation of the serotonergic neurons, and the consequent release of serotonin, is facilitated in dominant animals and suppressed in subordinates, probably as a result of input from higher centers in the CNS.

The vertebrate nervous system is significantly more complicated than the invertebrate nervous system, and it is much more difficult in vertebrates to associate specific behavioral functions with particular neural networks. However, research suggests that in mammals, too, the performance of aggressive behavioral patterns, and the modulation of an animal's tendency to fight, are controlled by a hierarchical system of neural structures. Many of these structures are found in the limbic system, that part of the forebrain involved predominantly with emotional behaviour and motivation. The aforementioned neural structures interact with biochemical produced both within and outside the nervous system. For example, in several vertebrate species, electrical stimulation of the midbrain and hindbrain elicits stereotyped and undirected patterns of aggressive behaviour, whereas stimulation of the hypothalamus and the nearby pre-optic region (both found in the forebrain) elicits well-

coordinated attacks on other members of the same species. Lesions in these areas reduce aggression. These and other observations imply that the hypothalamus and the pre-optic area of the forebrain are involved in the generation of coordinated aggressive behaviour that are, in turn, produced in lower brain regions. The activity of this system is modulated by higher centers, including areas of the limbic system—specifically the septum, which lies above the hypothalamus and has an inhibitory effect on aggression, and the amygdala, found deep in the temporal lobes and having the opposite effect.

The limbic system is rich in neurons containing serotonin and norepinephrine. Observations suggest that high levels of serotonin are associated with reduced aggressiveness and that high levels of norepinephrine are associated with increased aggressiveness. In a range of vertebrate species, fighting experience has a marked effect on brain biochemistry, especially on the limbic system. For example, in rainbow trout and in lizards, dominant animals show transient activation of the brain serotonin systems, whereas subordinates show longer-term elevation of these systems.

Factors affecting aggression

A) Internal factors:

a. Limbic system – Hypothalamus is involved in defence and escape behaviour e.g. pigeons, cats, monkeys.

b. Hormones – Neurosecretion and hormones like epinephrine are related to physiological arousal. Dopamine or serotonin also may affect aggressiveness. Testosterone also makes males more aggressive during breeding. E.g. elephants 'Mast' stage.

The influence of testosterone

Many vertebrate brain structures involved in the control of aggression are richly supplied with receptors that bind with hormones produced in the endocrine system, in particular with steroid hormones produced by the gonads. In a wide range of vertebrate species, there is a clear relationship between a male's aggressiveness and his circulating levels of androgens such as testosterone, a hormone produced in the testes. From fish to mammals, aggression levels rise and fall with natural fluctuations in testosterone levels. Castration has been found to reduce aggression dramatically, while experimental reinstatement of testosterone—for instance, through injection into the blood—restores aggression. Circulating testosterone can even influence the structures and signals used during fights. In stags the neck muscles needed for effective roaring enlarge under the influence of rising testosterone levels. In male mice the scent of another male's urine, which contains the breakdown products of testosterone, elicits intense aggressive responses.

The close link between aggression and testosterone is not surprising, given that males of many species fight over access to fertile females, but the connection is complex. For instance, the more elaborate the social structure of a species, the less drastic are the effects of castration on aggression. In addition, testosterone of nongonadal origin (i.e., produced by the adrenal gland) may be important in aggression outside the breeding season, as in the case of birds such as the song sparrow that maintain nonbreeding territories in the winter. Furthermore, hormones other than testosterone and its derivatives also may be involved in the modulation of aggression. For example, in several species of mammals and birds, the distribution of the neuropeptide hormones arginine vasotocin (AVT) and arginine vasopressin (AVP) in the pre-optic and septal regions of the brain differs between the sexes. Aggression in males is facilitated by implants of AVT in the limbic system and inhibited by implants of AVP. Finally, while a causal link between circulating testosterone levels and aggression has been

well established, it is also clear that the link can work in the opposite direction, with participation in a fight having rapid effects on hormone secretion. In particular, many vertebrates that win fights show increased testosterone levels, while losers exhibit not only reduced levels of testosterone but also elevated levels of the stress hormone cortisol. Changes in hormonal levels in turn modulate future aggressiveness. Such multiple and multidirectional links between brain biochemistry, circulating hormone levels, and aggression are a key part of the mechanisms whereby behaviour in conflict situations is adapted to both past experience and current circumstances.

c. Physical Factors: Epilepsy, dementia, psychosis, alcohol abuse, drug use, and brain injuries or abnormalities can also influence aggression.

B) External factors:

a. Learning and experience – Researchers generally accept that some external factors trigger the aggression in animals. Previous experience can produce semi-permanent changes in the individual and agonistic behaviour is exhibited accordingly as dominant or submissive.

b. Pain and frustration – Noxious stimuli such as noise; injuries etc. cause individuals to attack each other or any objects. Frustration due to limited resources and the high crowding may trigger disruption of the limbic and hormonal control and the individuals become aggressive. Such instances are common in aggregated animals.

c. Xenophobia – crowding (strangers, group size), breeding (cats) and feeding activity (fishes cannibalism) exhibit aggression normally during limited resources are available.

Restraint of aggression

1. Displays – aggressive displays like large antlers of deers, explicit coloration in fish and birds, postures and gestures in fish, mammals, are used to convey aggressive and to avoid conflicts. This behaviour has importance to avoid unnecessary killing or wounding.

2. Evolutionary – aggression and threats take less energy and physical combat are advantageous to individuals and the phenomenon has taken ages in the evolutionary course.

3. Social control and disorganization – Cichlid fish has shown that when strange fish of the same species are continually introduced in the group, fighting remains at a high level. So introduction of strangers disrupts the social control. In other instance when the male or female dominant *Rhesus* monkey dies or leaves the group, aggression within group increases called as social disorganization.

Cannibalism:

- **Cannibalism** is the act of one individual of a consuming all or part of another individual of the same family as food. To consume the same species or show cannibalistic behaviour is a common ecological interaction in the animal kingdom and has been recorded for more than 1,500 species. Human cannibalism is well-documented, both in ancient and recent times.
- Cannibalism seems to be especially prevalent in aquatic ecosystems, in which up to approximately 90% of the organisms engage in cannibalistic activity at some point in their life cycle. Cannibalism is also not restricted to carnivorous species, but can also be found in herbivores and detritivores.

Types of Cannibalism

- **Survival Cannibalism:**

In the wild, the animals begin their struggle for survival. They start by eliminating their siblings, thus ensuring themselves of an extra helping of food. Cannibalism is also used to display dominance over other species at times.

e.g. - The golden eagle usually lays two eggs that hatch a few days apart. The one that hatches first turns out to be the stronger one, grabbing on to most of the feed that comes their way. In cases of extreme food shortage, the stronger one does not hesitate in making a meal out of its weaker sibling.

- **Sexual Cannibalism:**

Sexual cannibalism as the consumption of a mate before, during or after copulation.

e.g. - Praying mantis. When the female mates on an empty stomach, she happily bites off her mate's head to kill those hunger pangs. However, this doesn't happen as often as it is portrayed to be. Praying mantises are also known to indulge in elaborate courtship rituals, with the males taking considerable efforts to woo the lady. In other instances, the males have been observed to be prudent enough to avoid courting a hungry lady.

- **Filial Cannibalism:**

Filial cannibalism is when the parents consume their young ones, or when the adults eat up members of their own species.

e.g. - It is commonly observed in teleost fish families, where the eggs are eaten for their nutritive value, and are seen as an immediate source of energy. It is sometimes done to declutter an unmanageable brood.

Functions and evolution of aggression

Group versus individual selection

The nature of animal aggression, in most cases animals fight over food, shelter, and mates or over territories where these can be found. Therefore, in functional terms, it is easy to explain why animals fight: they do so to gain access to valuable resources. A more difficult question to answer is why conflicts are often resolved conventionally, by displays and threats, rather than by out-and-out fighting. For example, why does a stag, instead of using its antlers in an all-out bid for victory, withdraw from a fight after an exchange of roars, thus leaving its rival in possession of a group of fertile females?

For a long time the generally accepted answer was that animals refrain from engaging in overt fighting because the high level of injury that this can cause is disadvantageous for the species as a whole. According to this view, conventional fighting evolved because groups whose members behaved in this self-sacrificing way did better than, and gradually replaced, groups in which individuals fought fiercely in their own interest. This “for the good of the species,” or group selection, explanation has been rejected by most biologists for two main reasons. The first is that in a group consisting of altruists who fought conventionally, an individual who broke the rules by fighting as fiercely as possible would inevitably win fights, gain resources, and leave many offspring—some of whom would inherit the non-altruist’s disposition toward fighting, thus passing on non-altruistic traits to more individuals of future generations. In this way natural selection at the level of the individual would be stronger than selective processes at the group level. Except in highly unusual circumstances, therefore, group selection simply does not explain why the majority of aggressive encounters are settled without recourse to overt fighting. The second reason why the theory has been rejected is that

conventional fighting can be explained easily once it is recognized that, in addition to bringing benefits to the winner, aggression imposes costs on both opponents.

Cost-benefit analysis

Current understanding of the functions and evolution of behaviour has been greatly influenced by the economic approach that is central to the discipline of behavioral ecology. In this framework, both the costs and the benefits of particular actions are determined, ultimately in terms of their Darwinian fitness, which is an individual's genetic contribution to the next generation (through production and rearing of offspring) compared with that of other individuals. The cost-benefit analysis is then used to predict how animals should behave during fights in order to maximize their net fitness gains. Thus, the actual behaviour of animals can be compared with the predicted behaviour to see if the positive and negative effects of fighting on fitness have been correctly identified. This is not to suggest that animals make rational calculations about the consequences of their behaviour. Rather, it is assumed that natural selection, acting over thousands of generations, has resulted in the evolution of animals that are able to adjust their behaviour to the circumstances in which fights occur, by mechanisms that may well be unconscious (like the neuroendocrine effects).

The positive consequences for fitness, gaining preferential access to food and shelter and acquiring mates, are easy to specify if not always easy to measure. The negative consequences (or costs) of fighting are not so evident, but they include expenditure of energy and loss of time that might be devoted to other activities. For example, male sparrows that continue to fight over territories after they have acquired a mate neglect the care of their young, which do poorly as a consequence and in a diverse array of species, from crabs to crickets to sage grouse, aggressive displays and intense fighting have been shown to increase rates of aerobic and anaerobic respiration and to deplete energy reserves. Additionally, an important cost of fighting is the risk of injury; the fiercer the fighting, the greater the risk. Putting these adverse effects into the cost-benefit equation has helped to explain many puzzling aspects of animal aggression. These include the fact that subordinate animals accept their low status that animals sometimes reduce the size of their territory or even abandon it altogether, and that, once a fight does get under way, animals do not always compete to the limit of their capability.

That subordinate animals accept their low status, even though by fighting they may ascend the hierarchy and gain advantages, can be explained in terms of the costs of fighting for the challenger. Subordinate animals are often small or young and are less likely to be able to challenge a dominant animal successfully. Since the fight is likely to be fierce and the risk of injury high, the costs of challenging outweigh the potential benefits of winning. Therefore, the individual fitness of a subordinate animal may be greater if it submits to a rival rather than launching a challenge. If the animals concerned must live in a group in order to survive, as is the case with wolves, then subordinate individuals may be "making the best of a bad job" by accepting long-term subordinate status. On the other hand, dominant individuals pay a high price for their status. Often challenged by rivals that are closer to themselves in size and strength, they must frequently engage in energetic and potentially dangerous fights, which may shorten their tenure as the dominant group member. For example, dominant red deer stags defending large groups of hinds end the breeding season in very poor condition, and they rarely retain their high status for more than a few years. Younger subordinate males, by keeping out of trouble until they become stronger and the dominant animal weaker, may actually increase their chances of ultimately achieving high status, with its accompanying benefits. Subordinate animals may even use tactics other than fighting to gain resources. For example, subordinate red deer stags sneak mating opportunities with fertile females while dominant males are busy fighting each other. In salmon, subordinate juveniles acquire food by foraging at times when

their dominant neighbours are satiated. Badges of status, such as the Harris sparrow's black throat and crown feathers, facilitate the process of establishing and maintaining stable hierarchical relationships because only dominant animals can afford to pay the costs of getting involved in fights. In the case of the sparrows, subordinate males whose stripes have been enlarged experimentally are attacked by larger or stronger birds against whom they cannot adequately defend themselves.

Territorial behaviour

Territorial animals sometimes reduce the size of their defended area or even abandon it altogether. e.g., during the winter, pied wagtails are often seen to switch between defending and sharing their feeding territories along riverbanks. Such flexible behaviour can be explained in terms of the shifting balance between the costs and benefits of fighting over space. In brief, animals will defend territories when the distribution of resources and the density of competitors make it economically advantageous for them to do so, but they will abandon territorial defense when this ceases to be the case. This can be seen most clearly in the context of feeding territories, where the benefits gained from ownership (energy taken in) are in broadly the same currency as the costs of defense (energy expended).

The simple graphs shown in the figure 3 illustrate the costs and benefits of defending territories of different sizes. The model assumes that the energetic costs of fighting increase exponentially with the size of the territory because the defended area of a circular territory increases as the square of its radius. It also assumes that the benefits gained level off at larger territory sizes because there is a maximum rate of feeding beyond which animals cannot utilize more food. (Other models assume different shapes for these two curves, thus altering the predictions.) The net gain (or cost) for each territorial size is measured by the distance between the cost and benefit curves, as shown in the figure. The optimum territory size is the one corresponding to the maximum distance between the cost and benefit curves, indicating maximum net gain.

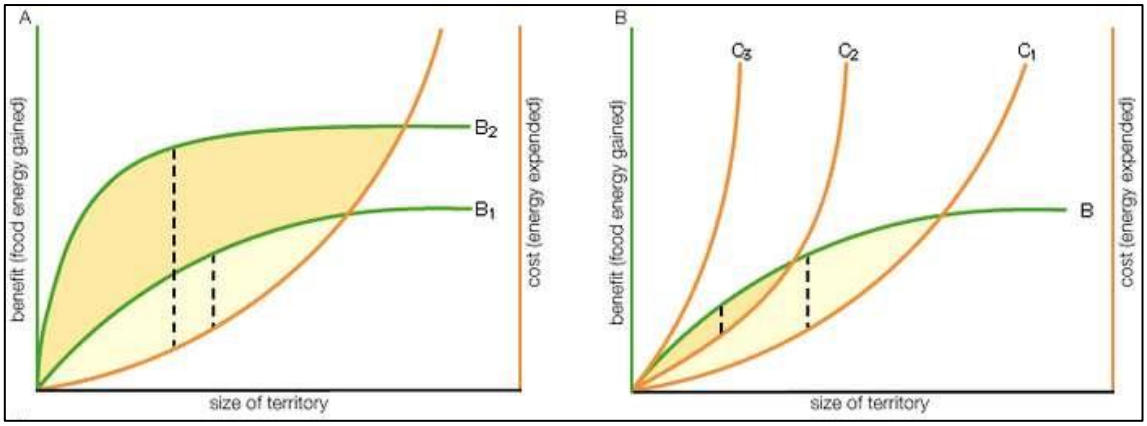

Fig 3: A cost-benefit analysis of territorial behaviour

Graph A shows that an increase in the density of available food (from B1 to B2) shifts the optimal territory size (i.e., the size that maximizes net gain) to the left, which means that owners should reduce the size of their territory. Thus, the model predicts that there should be a reduction in territory size in response to increased availability of food within it—a prediction shown to be true for species ranging from limpets to trout, hummingbirds, and squirrels. On the other hand, the cost of defending a territory of a given size can change; for example, it may increase as the number of individuals competing for a given patch increases.

Graph B in the figure shows that the size of a territory for which the benefits of ownership outweigh the costs of defense (i.e., there is net gain) becomes smaller as the cost of territorial defense increases from C1 to C2. Eventually a territory of any size ceases to be economically defensible (i.e., when C2 increases to C3). Therefore, the model predicts that territorial defense should be abandoned when a certain level of cost has been exceeded. Such an effect has been described for a variety of animal species, including migrating sunbirds defending patches of nectar-rich flowers and salmonid fishes defending feeding sites in streams.

Competition

Competition is a relationship between organisms in which one is harmed when both are trying to use the same resource related to growth, reproduction, or survivability. Competition stems from the fact that resources are limited. There are simply not enough of some resources for all individuals to have equal access and supply. Competition can occur between organisms of the same species, or between members of different species.

Competition between species can either lead to the extinction of one of the species, or a decline in both of the species. However, this process can often be interrupted by environmental disturbances or evolution, which can change the rules of the game. Competition is often involved when species are limited in their range, often by direct competition from other organisms.

Intraspecific Competition

Intraspecific competition is a *density-dependent* form of competition. "Intra" refers to *within* a species, as opposed to "inter" which means *between*. Intraspecific competition can be summed up in the image below (Fig 4).

Fig 4: Intraspecific competition

In this image, two wild dogs known as *Dholes* fight over a carcass. The carcass is a *resource*, something both organisms need to survive. Intraspecific competition is density dependent for one reason. The more *Dholes* you have, the less food each one gets. To the individual *Dhole*, food is everything. With very few predators of their own, the most successful *Dholes* (the ones who survive and reproduce the most) often are simply the ones who eat the most.

Thus, while these *Dholes* may have coordinated to take down this deer, they are now competing to see which one will get to eat first. The one that eats first will get more, and be more likely to survive and

reproduce. The other one (or the last one if there are many) will not get as much. This will lower its survivability and the chances it will get to reproduce. Since evolution relies mainly on which organisms reproduce, this form of competition can quickly lead to changes in a population if only a few of the individuals are surviving and reproducing.

Interspecific Competition

Interspecific competition is between individuals which are different species. This could be between any two species, as long as they are competing over a resource. An interesting example of interspecific competition is found in coastal marine environments, like the coral reef in the picture below (Fig 5).

Fig 5: Interspecific competition

In this picture, there are dozens of species. There are several species of fish. Behind them, as a backdrop many people would ignore, is a canvas of dozens of species of coral. Coral, while it may look like some sort of rock or plant, is actually a colony of tiny animals. These tiny animals filter organic material from the water, and use stored bacteria to photosynthesize sunlight for additional energy. Thus, each coral species is competing with not only the other corals, but also with the fish for available nutrients and sunlight.

While corals might not seem like a competitive bunch, they are actually directly competitive with other corals. When an enemy coral is encroaching on their space, they can deploy chemical warfare to counter their rival. Often, coral fights end in one of the corals being killed by the other. While the corals are not *predators* of each other, the competition still ends in the death of one of the corals. The victorious coral was simply fighting for the resources it needs.

Direct and Indirect Competition

There is also another aspect of competition that can be applied to scenarios of limited resources, and that is the idea of direct vs indirect competition. Direct competition is like both of the scenarios above, and there are many more examples of it. Any time two or more animals fight or have a symbolized confrontation, this is probably some sort of competition for a resource.

However, *indirect competition* is when the two animals do not interact, but the presence of both animals in the same territory causes the competition. Think of the fish in the example above. If those fish feed on the same resources used by the corals, then the fish are in competition for the limited resources. Coral, being more or less anchored to the ocean floor, have little chance of directly

attacking the fish. Instead, this would be referred to as an *asymmetrical indirect competition*. The fish eat as much of the food as they want, and the coral are limited to scraps. The coral have no way of competing. Luckily for most coral reef systems around the world, the ocean has plenty of food for most.

Outcomes of Competition

Competition is not a static process. Once set in motion, it can go a number of different ways. While the models may show that it will eventually drive one species to extinction, in reality a number of things can happen. First, an environmental disturbance, such as a fire or large wave, can upset the ecosystem and destroy the advantage the best competitor had. Typically, a pinewood forest is made mostly of pine trees because they are the best competitors in the environment. However, after a forest fire the most populous plants are small, opportunistic plants that grow quickly. The fire causes a change in the environment, which completely changes the dynamics of competition.

Further, most competition is also an evolutionary pressure on both parties. Animals from both sides that compete the best are able to survive and reproduce. Thus, over time the competition tends to resolve itself. More often than not, the competition can devolve as the species adapt to use different resources or change the way it uses a resource. This is known as *character displacement*. It is most well-documented in finches. When two different species of finch live on separate islands, their beaks are the same size because they prefer similar seeds. When they occupy the same island, one of their beaks gets smaller while the other gets larger. This separates the resources they consume and alleviates the competition.

The niche concept

A species' **niche** is its ecological role or "way of life," which is defined by the full set of conditions, resources, and interactions it needs (or can make use of). Each species fits into an ecological community in its own special way and has its own tolerable ranges for many environmental factors. For example, a fish species' niche might be defined partly by ranges of salinity (saltiness), pH (acidity), and temperature it can tolerate, as well as the types of food it can eat.
[Is that the only way to define a niche?]

As we'll see, two organisms with exactly the same niche can't survive in the same habitat (because they compete for exactly the same resources, so one will drive the other to extinction). However, species whose niches only partly overlap may be able to coexist. Also, over long periods of time, they may evolve to make use of more different, or less overlapping, sets of resources.

The Competitive Exclusion Principle (Gause's Law of competitive exclusion)

To explain how species coexist, in 1934 G. F. Gause proposed the competitive exclusion principle: species cannot coexist if they have the same niche. The word "niche" refers to a species' requirements for survival and reproduction. These requirements include both resources (like food) and proper habitat conditions (like temperature, pH). Gause reasoned that if two species had identical niches (required identical resources and habitats) they would attempt to live in the exact same area and would compete for the exact same resources. If this happened, the species that was the best competitor would always exclude its competitors from that area. Therefore, species must at least have slightly different niches in order to coexist.

Peter Grant and colleagues tested Gause's principle by studying seed-eating finches (birds) that live on the Galapagos Islands in the Pacific Ocean. They found that different finch species can coexist if they have traits that allow them to specialize on particular resources. For example, two finch species, *Geospiza fuliginosa* and *Geospiza fortis*, vary in a key trait: beak size. Beak size is a critical trait because it determines the size of a seed that a finch can eat: Individuals with small beaks eat small seeds, individuals with intermediate sized beaks can eat intermediate size seeds and individuals with large beaks can eat large seeds. *G. fuliginosa* and *G. fortis* do compete for intermediate sized seeds because each species has some individuals with intermediate sized beaks. However, *G. fuliginosa* specializes upon smaller seeds because it has more individuals with small beaks. Conversely, *G. fortis* specializes upon larger seeds because it has more individuals with large beaks. Thus, these species niches differ slightly because a specific trait, beak size, allows them to specialize upon a particular seed size.

Joe Connell also tested Gause's principle by studying barnacles (shelled marine organisms) that live on rocks along European coastlines. In 1961, Connell found that two barnacle species, *Balanus* and *Chthamalus*, can coexist because they differ in two traits: growth rate and vulnerability to desiccation. *Balanu* 's growth is rapid, which allows it to smother and crush the slower-growing *Chthamalus. Balanus,* however, dies close to shore because it gets too dry during low tide. In contrast, *Chthamalus* tolerates these dry conditions. Consequently, even though *Balanus* is a better competitor for space, these barnacles coexist because *Chthamalus* can survive in areas that *Balanus* cannot survive. These and many other examples support the competitive exclusion principle: Species can only coexist if they have different niches.

Dominance

Dominance in the context of biology and anthropology is the state of having high social status relative to one or more other individuals, who react submissively to dominant individuals. This enables the dominant individual to obtain access to resources such as food or access to potential mates, at the expense of the submissive individual, without active aggression. The opposite of dominance is submissiveness. Dominance may be established by fighting, or merely by threatening displays or interchanges; once established, however, dominance relationships may reduce the level of aggression between the individuals concerned.

Dominance may be a purely dyadic relationship, in which case the fact that individual A is dominant over B has no implications for whether or not either of them is dominant over a third individual C. Alternatively, dominance may be hierarchical, with a transitive relationship, so that if A dominates B and B dominates C, A always dominates C. This is called a linear dominance hierarchy or pecking order.

In hierarchical societies, the dominant individual in a group may exert control over others; more commonly, however, decision-taking about the actions of the group is dissociated from social dominance.

In animal societies, dominance is typically variable across time (as individuals age, gain or lose social status, or change their reproductive condition), across space (in territorial animals, territory owners are dominant over all others on their own territory but submissive elsewhere) or across resources. Even with these factors held constant, perfect Dominance hierarchies are rarely found in groups of any size (at least in the wild; dominance hierarchies may be more frequently found in captivity, since they tend to be induced by focused resources such as limited supplies of food supplied in a fixed place). Nonetheless, there are some species in which clear dominance hierarchies are seen, and in any

case establishing the dominance relationships between individuals is the usual first step in describing the social relationships within any animal group.

Dominance is a fact. Nonhuman (and human) animals dominate one another in a number of ways. Individuals may dominate or control

(1) access to various resources including food, potential and actual mates, territory, resting and sleeping areas, and the location in a group that's most protected from predators;

(2) The movements of others; or

(3) The attention of others.

Even if dominance interactions are rare, they do occur, and that is why it's important to log many hours observing known individuals. As one gets to know individuals in a group he or she also learns more and more about the subtle ways in which a wide variety of social messages are communicated, including those used in interactions in which one individual controls another.

Infanticide

Infanticide is any behaviour that makes a direct and significant contribution to the immediate death of an embryo or newly hatched or born member of the pardoner's own species. Two principal types of infanticides can be distinguished; viz., non-kin infanticide and kin infanticide. The latter type results, in sacrificing shared genes for some presumed compensating benefits to the perpetrator's inclusive fitness. Kin infanticide can be further divided into two categories, viz., parental infanticide (done by the parent) and siblicide (done by a sibling, and sometimes called fatricide or cainism).

S.B. Hardy (1979) has classified infanticide in animals into five categories, viz., exploitation (i.e., cannibalism), resource competition, sexual selection and social pathology. Direct contribution to infanticide indicates that either plain aggression or abusive neglect, or both, are involved. But the death need not be caused by any particular blows. Thus, aggressive intimidation of a nest-mate as an infanticidal act (i.e., siblicide) is included if, as a consequence, the victim starves to death, but the starvation would not be regarded as infanticide if it resulted from aggressive sibling competition.

Infanticide and Cannibalism

Infanticide may or may not lead to cannibalism. Several cases are known in the animal world where cannibalism is the end and apparently the only purpose of infanticide, and it is not clearly distinguishable from predation; here we also include 'ovicide', the killing (and eating) of eggs by conspecifics. Many fishes and insects eat their eggs and young ones.

A recent example is that given by Roonwa J. and Rathore (1975) who observed deacylated brooding termites, microtermes microphagous of the Indian Desert, eating young larvae in the colony: "alates often attack and eat up young larvae, a process which is finished in about 5 minutes. Sometimes the larvae are merely bitten and then left over in dead or dying condition"; it was not possible to say whether the cannibals included the parents of the larvae. Similarly, I have seen the Old World Desert Locust (*Schistocerca gregaria*) attack and eat the older (fourth and fifth stage) hoppers while they were undergoing moulting; it is possible that the hoppers were eaten not merely for the flesh but also for water. Steiner (1972) reported ground squirrels eating conspecific infants. I have seen a mother rat, *Rattus rattus*, eating young infants front her own litter, and a bitch eating a member of her own litter a few hours after giving birth, in both can apparently due to hunger.

Infanticide in Fishes:

In fishes intraspecific predation of young stages (eggs, embryos, larvae and juveniles) is of common occurrence. Here, infanticide always results in cannibalism which is far more common than in the terrestrial vertebrates. Several factors have contributed to this situation. Among them, the more important ones are the following:

(i) High fecundity, and the resulting smaller' offspring;

(ii) The predominance of external fertilization, resulting in the deposition of helpless embryo in a potentially hostile environment; and

(iii) Filial cannibalism, which is favoured by the frequent occurrence of parental care of offspring by males in contrast to the general prevalence of care by the mother in most other animal groups (Dominey and Blumer 1984).

Infanticide in the Amphibia:

In the Amphibia to the highest mortality occurs in- the younger stages (eggs and larvae), and the problem has been reviewed by Simon (1984). This is due to various causes, e.g., predation by the vertebrates and the invertebrates, fungal and other infections, competition, disease, starvation, hostile environment (extremes of temperature), etc. In some salamanders and frogs the father guarding the eggs is said to eat them occasionally (filial cannibalism).

Infanticide in Human:

Human infanticide can take many forms ranging from outright physical killing of the infant to its neglect, abuse, etc. It includes destroying the foetus by abortion, or by killing it outright by piercing the abdomen with a sharp instrument, etc. The infant may be drowned in water, placed in a basket and floated down the river; left alone in a jungle or the middle of a desert to die of starvation or be killed by predators. The subject has been reviewed by several authorities, notably Chandrasekhar (1959, India), Dickeman (1975, 1984), S.B. Hardy. (1979), Harris (1977), Williamson (1978), Scrimshaw (1984), Johansson (1984), Daly and Wilson (1984) and Bugos Jr. and McCarthy (1984).

Infanticide is found in all societies, from the most primitive to the most civilised, throughout the world and over the centuries. Earliest man, in the hunter-gatherer, horticulturist and early agrarian societies, practised it for supposed purposes as diverse as population control and maintenance of the social structure (Dickeman 1975). In London in the 19th century: infants were commonly left abandoned in parks and ditches to die, and similar practices to kill infants were found in many parts of Europe (Scrimshaw 1984). Although infanticide seems to have disappeared from most parts of the world, it is said to be still practised in China. As stated in a large number of cases, human infanticide can be caused in a variety of ways, viz., deliberate killing (sometimes in rituals to propitiate a god, goddess, or to obtain kingship), placing the child in a dangerous situation, "accidental" death, excessive physical punishment, and lowered biological support. Whatever the immediate method or methods used for infanticide, the practice has certain limited biological 'consequences. Among the most obvious of these is population control, particularly where the infant killed is a female. On the whole, however, it may be pointed out that infanticide in man was on so small a scale, compared to the total population in an area, that it probably did not have any large-scale biological consequences, and the various theories advanced to account for infanticide (particularly, comparisons with the non-human primates and other animals) are mostly idle speculations. Infanticide in man is simply a case of social pathology.

Theories of infanticide

a) Establishing social bonds :

This theory was advanced by Sugiyama (1964, 1965a, b, 1966, 1967) on the basis of his observations on the langurs (*Presbytis entellus*) in Dharwar, Southern India. He noted that the loss of the infant stimulated the onset of oestrus in the infants' mother. This situation helped in establishing social bonds between the group of females and the new male.

b) Sexual excitement of males:

Mohnot (1971), who was the first author to eye-witness infant killing, by males In the Hanuman langur, *Presbytis entellus*, in Jodhpur (India), stated that infanticide was induced by sexual excitement. He wrote "The aggressive attitude of the attackers, their sexual excitement (with the erection of the penis), their probable influence on the oestrus of the females, presentation by the oestrus females towards the invader, subsequent copulations by them and their overstay with the sexually excited females indicates that the probable motive behind the attacks has some relationship with the sexual urge of the 'males. " All the infant deprived females came into oestrus quickly (within 6 days of the death of the infant), and mostly copulated with the killer males.

c) Xenophobia:

The killing of conspecific strangers is widespread among animals and has been reported to occur in several groups of animals, e.g., insects, fowl, geese, wolves, hyenas, primates and man. For example, in the Indian termite, *Anacanthotermes macrocephalus*, when two workers from different colonies are brought together, they often attack each other viciously, and within minutes one of them is seen lying fatally wounded or even cleanly decapitated (Roonwal, personal observations).

d) Dominance assertion:

From inferred cases in the Hanuman langur, *Presbytis entellus*, Parthasarathy and Rahaman (1974), postulated that infanticide was committed by the male to assert. His dominant status. In the red howler monkey, *Aloutta seniculus*, Crockett and Sekulic (1984), who analysed several inferred cases of infanticide, concluded that the infant killers were new or newly dominant males.

e) Sexual selection:

The sexual selection theory was proposed by S.B. Hardy (1974, 1977) to account for infanticide in the Hanuman langur, *Presbytis entellus*, and postulated that it evolve as a result of male-male competition for gaining reproductive advantage. Following infanticide, the mother often comes into oestrus quickly and copulate with the killer male, a situation which provides support to the theory. On the other hand, the expectation that the attacking male attacks and kills only those infants who were not sired by him, is seldom proved for the simple reason that he cannot possibly recognise his own offspring (even man cannot do so).

g) Resource competition:

On the basis of observations in the New World howler monkey, *Allouta seniculus*, Rudran (1979 a,b) concluded that increasing male fitness through the reproductive strategy of infanticide (Hardy's Sexual Selection Theory, supra) is not a primary reason of infanticide but merely a secondary consequence. Infanticide is primarily an extension of the process of eliminating unrelated food competitors who do not benefit the invading male in any way. Regulation of population growth is another consequence of infanticide. According to Rudran, the differences in the ratios of benefits to

cost of the process may explain the observed differences in this pattern of social change between the red howler monkey (*Alouatta seniculus*) and other primates (e.g., the langur, *Presbytis entellus*).

h) Population control (density-dependant infanticide) and prevention of inbreeding:

On the basis of data from the Hanuman langur, *Presbytis entellus*, in Dharwar, Sugiyama (1967) suggested that infanticide is the product of high population densities and is a possible mechanism for population control; Eisenberg et al (1972) came to the same conclusion. In man, infanticide probably serves as a means of population control and prevents inbreeding (Dickman 1975, Ripley 1979, 1980). The device serves as a means of ensuring genetic polymorphism in the, tangurs also where the social structure and breeding pattern favour inbreeding (Seger 1977). Boggess (1984) has brought forward several factors which go against this theory.

I) 'Following response' induction:

Pirta (1981) emphasised that a wide range of behaviour patterns are employed by males to induce a "following response" in females. A male's glance and movement are sometimes enough for this purpose during consort relations. At other times, a male may clasp, pull and bite a female, as in, the hamadryas baboon, *Papio hamadryas* (Kummert and Kurt 1965). In the Olive baboon, *Papio anubis*, the male stole an infant from a female several times to induce a following response. In extreme cases, a male wounds (usually by biting) or even kills an infant, as in the-Hanuman langur (*Presbytis entellus*) or in the Rhesus macaque (*Macaca mulatta*).

j) Parental manipulation:

Infanticide in traditional human societies, in contrast to other primates, appears to be primarily a sort of parental manipulation of their progeny to achieve various ends (Alexander 1974, Dickman 1975, Roonwa11977). The death of an infant and the termination of parental investment will sometimes improve the chances of survival of either the mother or ber older offspring, or will increase the reproductive fitness of both the parents (Hardy and Hausfater 1984).

Probable questions:

1. Write short notes on sexual selection. Give examples.
2. What is intersexual selection and intrasexual selection?
3. Differentiate between intersexual selection and intrasexual selection.
4. Elaborate Bateman's principle.
5. What is aggression?
6. Discuss different types of aggression.
7. What is impulsive aggression?
8. Discuss various forms of aggression with suitable example.
9. Mention the purposes of aggression.
10. Discuss the role of neuroendocrine influences in aggression.
11. Discuss on internal factors affecting aggressive behaviour.
12. What is xenophobia?
13. What is cannibalism?
14. Discuss different types of cannibalism.
15. Write a detailed discussion on cost-benefit analysis of territorial behaviour.
16. What do you mean by interspecific competition and interspecific competition? Give example.
17. Elaborate the Gause's Law of competitive exclusion.
18. What is the relationship between infanticide and cannibalism?
19. Discuss different theories of infanticide.

Suggested Readings:

1. Alcock, J. (2001). Animal Behaviour: An Evolutionary Approach. , Sinauer Associate Inc., USA.

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4. Mandal, F. (2010). A Text Book of Animal Behaviour. Pentice Hall India.

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6. Refinetti, R. (2000). Circadian Physiology. CRC Press, Boca Raton.

7. Ruhela, A. and Sinha, M. (2010). Recent Trends in Animal Behaviour. Oxford Book Co. Jaipur.

8. Smuts, B.B. and Smuts, R.W., 1993. Male aggression and sexual coercion of females in nonhuman primates and other mammals: evidence and theoretical implications. *Advances in the Study of Behavior*, *22*(22), pp.1-63.

Unit V

Communications: Channels, functions, origin and modification of signal, signal receiving mechanism

Objectives: In this unit you will know about Communications; more preciously channels, functions, origin and modification of signal, signal receiving mechanism.

Introduction

Communication

Communications is fundamental to the existence and survival of humans as well as to an organization. It is a process of creating and sharing ideas, information, views, facts, feelings, etc. among the people to reach a common understanding. Communication is the key to the directing function of management. A manager may be highly qualified and skilled but if he does not possess good communication skills, all his ability becomes irrelevant. A manager must communicate his directions effectively to the subordinates to get the work done from them properly.

The widest includes any method by which one animal or cell demonstrably influence the behaviour of another (Wilson, 1975), is known as communication.

It is not difficult to select examples of behaviours which obviously qualify as some sort of communication - such as warning cries, or the release of chemical attractants. There is little agreement, however, on a formal definition to cover less obvious cases. The widest includes any method by which one animal (or cell) demonstrably influences the behaviour of another (Wilson, 1975). This is straightforward enough, but it is usual to qualify it in ways related to intuitive notions of functional specialization. The natural world is sustained by one animal eating another: an act of this kind undoubtedly alters the behaviour of both, but is certainly not primarily communicative. Predation can easily be excluded, and for most purposes it is convenient only to consider interactions between members of the same species, although symbiotic relations between different species, and deception of predators by prey, may involve interesting specializations. The behaviour is reasonable to suppose have been selected because of the benefit too both the sender and receiver of the transient messages thus conveyed.

An animal respond to stimuli send out by another. The one who sends out stimuli is the ‘Signaller’ and one who receives it and respond accordingly is the ‘receiver’. There it is a cooperative transform of information from signaller to receiver which is called ‘Communication’.

Thus communication always involves the passage of information. Communication is adaptive for both the sender and receiver of the signal who change information perhaps concerning the location of food sources, presence of threat or their readiness to mate.

Communications Process

Communications is a continuous process which mainly involves three elements viz. sender, message, and receiver. The elements involved in the communication process are explained below in detail:

1. Sender

The sender or the communicator generates the message and conveys it to the receiver. He is the source and the one who starts the communication

2. Message

It is the idea, information, view, fact, feeling, etc. that is generated by the sender and is then intended to be communicated further.

3. Encoding

The message generated by the sender is encoded symbolically such as in the form of words, pictures, gestures, etc. before it is being conveyed.

4. Media

It is the manner in which the encoded message is transmitted. The message may be transmitted orally or in writing. The medium of communication includes telephone, internet, post, fax, e-mail, etc. The choice of medium is decided by the sender.

5. Decoding

It is the process of converting the symbols encoded by the sender. After decoding the message is received by the receiver.

6. Receiver

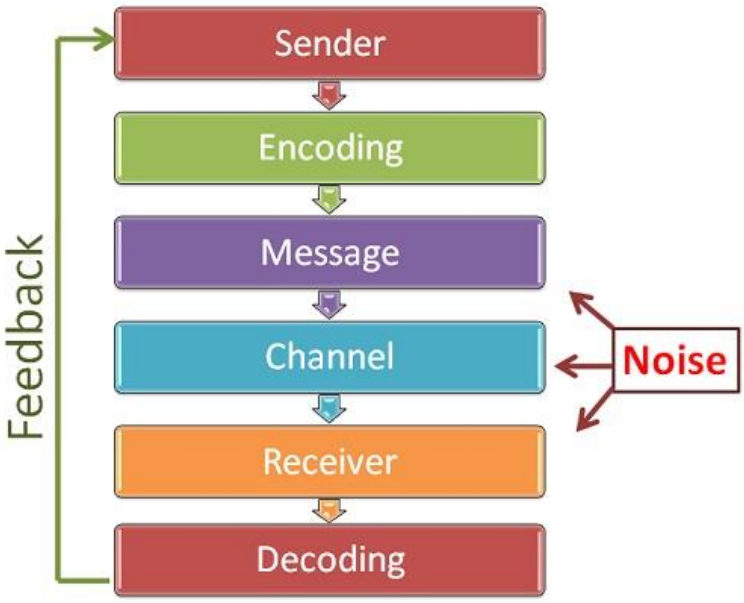
He is the person who is last in the chain and for whom the message was sent by the sender. Once the receiver receives the message and understands it in proper perspective and acts according to the message, only then the purpose of communication is successful.

7. Feedback

Once the receiver confirms to the sender that he has received the message and understood it, the process of communication is complete.

8. Noise

It refers to any obstruction that is caused by the sender, message or receiver during the process of communication. For example, bad telephone connection, faulty encoding, faulty decoding, inattentive receiver, poor understanding of message due to prejudice or inappropriate gestures, etc.



Components of communication

In the simple communication system, a sender encodes and transmit a signal which is detected by receiver who decodes the signal into meaningful terms. The components are –

i. *Sender* - This is sending a message.

ii. *Message* - It is designed communication as information.

iii. *Signal* – It is the physical import of the message.

iv. *Code* – Set of specific language in the signal.

v. *Receiver* – target of the signal.

vi. *Meaning* – is what the receiver makes out of the signal.

Effective of communication is may be influenced by many factor.

1. Error in receptor that may occur as result in
2. Error in detection may also occur in 2 basic ways –
    a. By missing some aspect of signal (missed detection).
    b. By reacting to some other stimuli which are not true stimuli (false alarms)

Influence of physical environment is great in effectiveness of communication. Fundamental problems of transmission of signal, particularly of sound which depends upon physical nature of habitat – attenuation or distortion.

Function of communication (or, why animal communicate)

The over-riding explanatory principle for animal behaviour is that of the theory of evolution, most often expressed not just in terms of the advantage of the individual, but by reference to the individual's genes. Behaviours under genetic control persist only if they optimise transmission of the individual's genes to the next generation. The initial presumption is that of selfishness, and this does not suggest that communicative mechanisms which provide mutual benefit should be at a premium. Communicative activities, if specialized, must entail costs of time, energy and computational effort to both sender and receiver: if both are selfish why do they not confine themselves to securing individual comfort and well-being?

General theories of social behaviour supply two kinds of qualification. The first is **kin-selection**. In genetic terms selfishness can encompass parental care, and indeed more extreme forms of self-sacrifice in return for the survival of sufficient numbers of close relatives. Observations of interactions between parents and young form a substantial part of the discipline of ethology - the approach to the study of animal behaviour which has supplied some of the conceptual framework for analyses of communication. The second qualification is "**reciprocal altruism**", which can be mathematically justified, but is suggested partly because of observations of elaborate social interactions not otherwise readily explicable. The most selfish individual should co-operate with others if its own benefits are increased as a result, which can happen if favours are returned, or if collective activities benefit all co-operators more than cheaters or individualists.

The direct relevance of these corollaries of evolutionary theory to the analysis of animal communication is that they both require some kind of individual and group *recognition*. If

parental care is to be justified by kin-selection it ought to be directed at the correct offspring: in relatively solitary species a minor constraint, but a major one in those breeding in colonies or flocks. Where group behaviour is co-operative, recognition of particular individual conspecifics is implied by the rules of reciprocal altruism, while where groups are formed as less complex aggregations, presumptively on "safety in numbers" grounds, there needs to be at least a sensitivity to whom else to aggregate with.

1. *Recognition*

A first function for communication is therefore recognition of species: more detailed social interactions for a variety of purposes need to be with conspecifics. Species recognition may be in many cases built in to sensory systems, but in some birds and mammals infants may acquire it from interactions with their parents, this process being known as *imprinting*. Acquired recognition is more obvious when local group membership is a factor in the details of communication, as for example with local dialects of vocalization (some song-birds) or local variations in group odour (some rodents). Within communities of social insects caste distinctions are typical, and within small groups of birds and mammals there is usually recognition of group members in terms of size, age and gender, and often also, as in the peck-orders of chickens and in other dominance hierarchies, recognition of each individual by any of the others. Generally it is assumed that animal communicative behaviours are genetically determined, and common to all members of the same species. Paradoxically however, it can be demonstrated in higher vertebrates, if not in insects, that one of the functions of the innate mechanisms is to enable individuals to acquire information which is peculiar to their own circumstances of life, which can include recognition of the identity of unique individuals, and membership of local groups. Even recognition of species membership may be learned, as with imprinting.

2. Courtship and mate selection

Even the most solitary species must engage in the social interactions required for sexual reproduction (parthenogenesis is extremely rare). Evolutionary theorists from Darwin himself onwards have deduced that mate choice is likely to produce via sexual selection effects not predictable from the more humdrum necessities of day-to-day survival. Hence the antlers of the stag and the tail-feathers of the male peacock, and the behaviours that accompany these. Dispersed species must communicate at a distance to bring males and females into proximity. Especially where several similar species co-exist in the same geographical area (not unusual for insects) fine distinctions between species-specific signals need to be made. Some species differences may be very straightforward, as with the different frequencies of calls made by species of cricket which differ in size. In other cases there appears to be more specialization in the evolution of particular signals, as in the patterning of acoustic messages in some other species of crickets, and more notably in bird song. The flashing of different species of firefly is also distinguished by temporal patterning. A further source of differentiation of species (and individual) recognition in mate selection lies in sequential "handshaking" between members of a pair. Male fireflies typically discriminate females of their own species by the precise time-delay between their own signal and the female's flash in response. Detailed sequential ordering of male-female interactions is common in courtship involving visual recognition, with an example being found in the ethologist Tinbergen's classic studies of the "zig-zag dance" during which a male stickleback leads a female back to his nest for spawning.

The degree of social interaction involved in mate selection varies from a single act of spawning to much more protracted shared parental activities, which are common in birds, where a male

and female often pair permanently, or for several seasons, in some species remaining together throughout the year, but in others separating outside the breeding period. Among mammals similar "pair-bonding" occurs in for instance beavers, when dam construction and/or maintenance is shared, in wolves and other canids, in which food is brought to pregnant or nursing females and young, and gibbons, where the functional reasons for the pairing are less obvious. Clearly methods of individual recognition, and signals for shared activities (e.g. turn-taking in incubation) are necessary in these cases. Sometimes the pair engage in elaborate gestural "ceremonies" during courtship, as in Great Crested Grebes, and these may contribute to both species and individual recognition via the visual modality. Individual recognition by smell is fairly standard in terrestrial mammals, but the use of all the sensory modalities available to any species is to be expected. Striking examples of vocal communication occur in species which use "dueting" or antiphonal singing between mated pairs. In East African shrikes, which forage together in dense foliage, each pair develops some idiosyncratic patterns among the several heard in antiphonal singing, while other patterns represent geographically localized dialects. Of seven species of pair-bonding lesser apes (gibbons and the Siamang) all but one have antiphonal singing between pairs (the other has lengthy all male and all female chorusing between adjacent groups). In two of these six species the male and female contributions never overlap. In the other four they do: in one species the male produces similar sounds to the female but at a lower pitch; but with the rest the male makes limited additions to a more complex female song.

3. *Aggression and Threat*

In deer and peacocks and many other species there is competition among males for access to females, along with male self-advertisement. Much but not all bird song is in this category. Aggression is of course also found in relation to disputes over territory and food. It is communicative in the specialized sense in so far as the interaction in the form of assertive calls and snarls, or gestural displays and postures, as alternatives or additions to direct physical struggle, which is usually the case. In highly social species including wolves and many monkeys' aggressive displays may be finely tuned according to the status of the interacting individuals in the group structure, and gestures of submission and appeasement from subordinates may be as important as those of threat from the more dominant. In these instances communication is usually over short distances, and indeed may include tactile sensation. Threat between neighbouring groups or between dispersed individuals usually involves greater distances and is well-served by loud vocalization, or territorial scent marking. It has been shown that bird song (even from a loudspeaker) deters other males from entering the vicinity of its location. The roars of the howler monkey can be heard a kilometre away, and may reasonably be assumed to have a territorial function. The elaborate chest-beating display of the male gorilla, which includes hooting, the shaking of branches and the slapping of the ground, is often directed at a neighbouring troop but is also probably related to the maintenance of dominance relations with closer observers.

4. *Alarm and Distress*

A more benign form of social signalling is anything which serves as a warning to others of danger from predators. There is an explanation for pure and simple social aggregation from the benefits to any single individual in terms of predator avoidance. But a selfish individual, on detecting danger, might seem best advised in the short term to make a disguised retreat, and indeed to maximize rather than reduce the disadvantage to its conspecific competitors. In groups with a high degree of consanguinity warning to others, or indeed self-sacrificial defence of them, would be explicable in terms of kin-selection, and this is obviously the case for the social insects

(which have single breeding queens for each community, and sterile workers; and, except for termites, have complicated increases in consanguinity because all males come from unfertilized eggs). Lesser degrees of consanguinity may contribute to the prevalence of alarm signals in small groups, particularly in monkeys and apes, where longevity, long-term group coherence and mutual dependence may foster reciprocal altruism. It has been suggested that in other cases giving an alarm call may in fact directly benefit the transmitting individual. That acoustic warnings (as opposed to alarms which elicit defensive aggression by the group) have evolved so as to be difficult to localize indicates that they are not without risk. However, it may to the advantage of an alerted individual to elicit collective evasive action - for instance, an individual bird in a ground feeding flock would probably be unwise to fly off on its own if it sights a hawk. Alternatively, signals given in reaction to predator proximity which are ostensibly altruistic may not be what they seem: it is now believed that the leaping ("stotting") of gazelles when fleeing advertises the vigour and alertness of the fleeing individual to the predator, rather than the presence of the predator to alternative prey. For whatever reason, apparently communicative reactions to predator presence are common, and are sometimes utilized by species other than that of the transmitter. They may differ in the same transmitting species, according to circumstances, including predator type. Mobbing calls in birds differ from other alerting or warning calls, in particular by being easy to localize, and the different cries made by vervet monkeys in response sightings of leopards, hawks, or pythons cause others to run to trees, to look up, or to look down, respectively. It is believed that infants learn the individual meanings of these calls, and experimental evidence suggests that fear of both natural and artificial objects is induced in both primates and birds if the young observe adults making alarm reactions to their presence.

Vocal or chemical signals elicited by fear or pain may function as warning messages, but as a special case, infant distress signals clearly function to elicit parental assistance. In experiments mother rats, cats, and hens react to the vocalization of a misplaced infant by taking steps to retrieve it (the specificity of the response in hens having been demonstrated by the fact that they ignore offspring visible distressed but acoustically isolated under a glass bell). Much less common than signs of distress in infants are "satisfaction messages" but the familiar purring of domestic cats, and similar sounds produced by only the young of wild carnivores, are sometimes given this description. As these sounds are accompanied by vibrations it is thought they may function to confirm bodily contact.

5. *Signals related to feeding*

The most celebrated example of animal communication is the system by which foraging honey bees convey to their fellows information about the location of recently used food sources (related bee species, and many species of ant, individuals lay olfactory trails to known food sources which are subsequently used by other colony members). But this kind of specific communication of the location of distant food sources is known only in the social insects. The most general form of communication in this category is simply the visual observation by one animal of another feeding, which has behavioural effects which are readily obvious in seagulls and other flocking birds. There is rarely any suggestion that the feeding animal emits signals with a specialized communicative role. However among social primates "food finding calls" have been identified, such as the low grunting of the chimpanzee when it discovers highly preferred food such as bananas or palm fruit. Food sharing occurs within chimpanzee groups and the rarity of food-finding signals is undoubtedly related to the fact that feeding is usually competitive. In chimpanzees and baboons there is occasional hunting of the young of other species by males, with its products being shared with females and infants, among whom there is

the intuitively understandable begging gesture of holding out the hand, palm upwards. In wolves and other canids meat sharing is routine in family groups, the reactions of the young, such as licking the muzzle of the returning parent, often being required to elicit regurgitation.
A similar pattern is very common in birds, and the interactions between parental feeding and the begging responses of nestlings was an early focus of attention for ethologists. A gaping response, with vocalization, occurs in the nestlings of many species, and the probability of being fed is related to the vigour of the begging. In gulls, nestlings peck at the parental beak to elicit regurgitation, and the precise visual features of the parent's head and beak which act best as stimuli for these pecks have been studied by the use of models. In fowl, where the young feed themselves from birth, there are usually special calls used by both parents to attract the young to food, and these are sometimes also included in courtship feeding of females by males.

6. *Co-ordination of group behaviours*

Flocking, herding and schooling require that individual animals influence one another, but these kinds of co-ordination may occur without the emission of specialized signals, as long as individuals are sensitive to the movements of others. Thus social facilitation is sometimes distinguished from co-ordination via known communicative mechanisms. However, in most social species, especially of birds and mammals, many more communicative behaviours are observed than can be accounted for in terms of the specific functions of mate selection, alarm calls and so on listed above. There are of course other functional categories, such as group migration and co-operative hunting, which have not been listed. Another area is juvenile play, in carnivores and primates, which is recognizable from specialized gestures, cries and facial expressions, and which is assumed to have some functions which are related to socialization. But there are also more general categories of behaviour which may serve vaguer functions such as social cohesion and affiliation. Thus species are said to use, for instance, "greeting displays" and "bonding messages" (vocal chorusing being an example of the latter). As the last point in this section on functions of communication therefore, one should note that although functionality is an underlying assumption it is by no means the rule that the functions of any agreed instance of animal communication can be readily identified.

Types of channels of communication

a. Tactile communication – is important in many animal. Touch is a very basic and faithful channel of communication but its score of transmitting information is limited. Social interaction of many invertebrates depend on tactile communication such as blind workers of termite colony which never leave their underground tunnel. Earthworm also rely on touch when they emerge from their burrow at night. Generally tactile communication operate only at closed range. Some animal has long antennae like cockroach and prawns who explore beyond their body length but those also has a limit. Some animals rely their effective length of touch by sensing mechanical disturbances of the medium by special chemoreceptors.

b. Visual communication – It is found in animal during many events and usual signal operate both at short and long range. Exhibition of immense and magnificent tail with numerous eye spot is a visual signal sent by peacock to peahen. Such a courtship signal operate at a closed range. Remarkable flashing of flies is also visual communication but operate over long distance.

When white tail deer (*Odoceolius sp*.) leap away sensing a potential danger, their tail is elevated, revealing their brilliant and also underside and white hairs of rump. This tail elevation is an alarm signal of white tail deer, an example of visual communication to fellow members of this group.

c. Acoustic Communication – It has been adopted by many insects, amphibians, birds that produce species specific acoustic signal trilling falls of male cricket are actually this signal to female announcing their readiness to mate. Bull frog males call to females by inplating and discharging air from their vocal sac. The females have no problem to distinguish a conspecific males call from a chorus of call in the same habitat. Songs of male birds are complex sounds with notes and phrase which are very species specific.

d. Chemical Communication – It is particularly important in insects, mammals. The chemical messengers known as pheromones are specific for species, used for communication individual of same sp. for various reasons including sex attraction. Female silk worm (*Bombyx mori*) produce pheromone form their gland associated with their reproductive system called Bombycol. Male silk moth contains numerous sensory receptor for Bombycol on the antennae. This receptor are so extraordinary sensitive to the stimuli that male can respond to a concentration of Bombycol as low as 1 molecule or 10^7 molecule on air. Workers are communicated with other workers are communicated with other workers of their nest by a trail of sent-which is chemical signal. Many larger mammals like tiger, deer, use chemical signal to announce the ownership to territory.

Importance of Communication

1. The Basis of Co-ordination

The manager explains to the employees the organizational goals, modes of their achievement and also the interpersonal relationships amongst them. This provides coordination between various employees and also departments. Thus, communications act as a basis for coordination in the organization.

2. Fluent Working

A manager coordinates the human and physical elements of an organization to run it smoothly and efficiently. This coordination is not possible without proper communication.

3. The Basis of Decision Making

Proper communication provides information to the manager that is useful for decision making. No decisions could be taken in the absence of information. Thus, communication is the basis for taking the right decisions.

4. Increases Managerial Efficiency

The manager conveys the targets and issues instructions and allocates jobs to the subordinates. All of these aspects involve communication. Thus, communication is essential for the quick and effective performance of the managers and the entire organization.

5. Increases Cooperation and Organizational Peace

The two-way communication process promotes co-operation and mutual understanding amongst the workers and also between them and the management. This leads to less friction and thus leads to industrial peace in the factory and efficient operations.

6. Boosts Morale of the Employees

Good communication helps the workers to adjust to the physical and social aspect of work. It also improves good human relations in the industry. An efficient system of communication enables the management to motivate, influence and satisfy the subordinates which in turn boosts their morale and keeps them motivated.

About signal

Origin of Signal:

A communication signal requires two activities (1) a producer capable of generating a receiver capable of detecting it and (2) integrate corporation that is require in communication arose.

One views that most animals are preadapted to generate signal because their normal everyday activity provides potential information. As for e.g. when a water strider walks on water on water surface, it creates small waves. It is now know that these waves carrying information about the presence of strider who generates waves to communicate with other.

Raw materials for communication signal may be provided by action of animals conflict behaviour may occur in animals, though it reduce frequency. When two rivals herring gulls meet at the border between their territory, they may attempt to attack and to escape from the opponent. The conflict may cause a male to show pecking behaviour to its opponent (without attack action) or may redirect attack to a set subject (Clump of grass) rather than rival male. Such conflict behaviour have been involve in the origin of certain stereotype behaviour.

Cost and Benefit of signal:

Adaptive value of communication, signal can be analysed by cost and benefit approach. This approach based on assumption that a specific trait with evolve and with spread through a population only when a benefit to its processors exceeds its cost by grater margin that another alternative forms of the trait. Usually such an assumption leads to the expectation that individual with transfer information to other only when they have reproductive interest.

Signal giving is a threat if it is an evolve adaptation, we can expect its benefits to be relatively high and its cost to be relatively low.

Male song birds sing in the spring, one bird may generates several thousand full throated song in a day. The clear substantial cost of singing are time and energy. Moreover, a singer may provide information through a predator about its location which can be great disadvantage to the singer.

There are straight forward alternative hypothesis on the benefit of signalling by singing. Because the singers are males, the songs are design to defend a breeding site against rival males if then at a distance- the male repulsion theory. Alternatively song may be designed to attract female through singers- Female attraction hypothesis. The experiment shows some male birds sing to defend territory, yet some sing to attract female, there is also evidence that males of some species do sing at a time when males are most fertile- Male guarding hypothesis. In all cases, singing in males increases their reproductive success.

Signal receiving mechanism

- ***Chemical Signals***

All forms of life must selectively detect and take in chemicals, and so chemical signaling occurs at many levels in all cells. Hormones operate within an organism, pheromones are signals between conspecifics, and allomones are intended for interspecies communication. In this section we will focus on pheromones.

Two different systems of reception are employed for chemical communication. Airborne and waterborne chemicals received at a distance from their source are detected by olfactory reception, or smell. Other chemicals require contact reception, direct contact of the receiver with the source of the

pheromone. Pheromones are usually produced by glands located on the skin, or by ducts within the body that have ducts leading to the body's exterior; the second variety of glands are known as exocrine glands. Mammals produce pheromones in the sudiferous (fluid-producing) gland and the sebaceous (waxy-substance producing) gland. Some mammals have other specialized glands. Insects produce a large variety of pheromones, most notably from their mandibular glands, thoracic glands, and stingers.

Some important examples of chemical signalling

A good example of a multipurpose pheromone is the Queen substance employed by some eusocial bees. This pheromone motivates and attracts workers, releases swarming, and is a sex pheromone. Absence of queen substance indicates the colony has grown too large (the queen is too far away to smell her) and so workers will build queen cells to rear new queens. An abrupt absence of Queen substance results in emergency queen rearing, since that absence is probably an indication of the queen's death.

Other examples of chemical signaling include alarm pheromones, such as bee stings. Isopentyl acetate, the chemical injected into a sting wound not only serves to wound an enemy, but to alert other bees to danger, and in some case cause swarming. The reason "killer bees" are so deadly is not because they have a more venomous sting, but rather because these bees have a lower threshold for alarm pheromone and so an entire swarm will react and sting the enemy to death. Ants and snakes employ trail pheromones to mark the path to a food source. These chemicals are laid out along a trail, and the next ant will follow the trail by means of contact reception. Many animals, from moths to cats, use pheromones to attract mates.

- ***Visual Signals***

Visual signals are limited because they require a direct line of sight and lighted conditions, and they only last as long as the sender is signaling. However, studies of communication have overemphasized visual communication, most likely because humans and primates are much more dependent on this type of communication than non-primate animals. We will not spend much time on visual signals because we are already familiar with them from our daily lives. The sender can send a signal by performing a display or by assuming a specific body posture. The receiver views the signal by means of eyes, which the brain translates into a visual image. Visual images are received in real-time, and so are generally dynamic signals.

Visual signals allow for a certain amount of cheating; that is, deceptive signals can lure receivers into responding to the benefit of the sender and the detriment of the receiver. Photuris fireflies are the only predatory species of firefly. By mimicking the female response of the prey species the "femme fatale" Photuris female lures in males, and then preys upon them. Wary males are careful in responding to female displays of their own species for fear of being preyed upon by the Photuris females. In this way, the prey males experience conflicting pressures from natural selection, which demands both individual survival and mating for species survival. This example reveals another problem with visual signals--they are not receiver specific. Any animal can potentially react to the visual signal of any species.

- ***Representational Information***

Most displays reveal information about the signaler, whether it be fitness, disposition, or location. Representational information imparts information about the environment external to the sender. This is a more complicated form of communication, as it requires first assimilating information about the environment, and then divulging that information to others. The honeybee dance language is an

example of representational information, imparting both the distance and the direction from the hive to food. A forager will return from a food source and, by performing a directed series of movements, can inform a second wave of foragers as to the location of the same food source.

- ***Acoustic Signals***

Acoustic forms of animal communication have greater superficial similarity to human speech than chemical or visual signals. Apart from this, this acoustic channel shares with olfaction the virtues of broadcast transmission over considerable distances, and availability in the dark or when lines of sight are interrupted. It is arguable that in many species (songbirds, crickets) hearing as a sense must be specialized for the purpose of social communication, as opposed to food seeking and predator avoidance. In others, by contrast, (bats and dolphins) the acoustic sense is utilized as the major avenue for prey detection - by echo-location. This is sometimes considered to be "solipsistic communication", but falls outside the present social perspective. Many other predators (cats, owls) have highly developed hearing which is used for detecting sounds produced by prey.

Acoustic signals are energetically costly, but can travel great distances, degrading with increasing distance. Many animals produce sounds to impart information, however only humans have a well-developed language. There is some evidence that Vervet monkeys have a language consisting of three distinct words: snake, eagle, and leopard. As it turns out, these alarm calls actually represent the type of threat, rather than the specific type of predator. The snake call warns conspecifics of the presence of a slow predator on the ground. Vervets respond to this call by standing up and looking around. The eagle call indicates a fast-flying predator. Vervets will run for cover and look up. The leopard call alerts the monkeys to a fast-running predator, and they respond by running up a tree.

- ***Tactile Signals***

Physical contact is limited in its ability to communicate because it is extremely short-range. Many invertebrates use antennae as the first line of contact with objects and organisms. The honeybee waggle dance used to explain the location of a food source is often performed in a dark hive, and so the foragers receive their information by interpreting the dance with their antennae. The most common use of tactile communication occurs during copulation. Tactile stimulation by males will often let a female know when to adopt a sexually receptive posture, as in rodents. In primates, grooming is an extremely important social activity. It functions to remove parasites, but also to secure social bonds. This is also true of humans, for whom touch is an intimate form of communication.

- ***Electrical Signals***

Sharks and some fish have electroreceptors that are used to detect objects and to socially communicate. Electro location is a form of auto communication; signalers send and receive their own signals. The difference between the emitted and received signals yield information about the environment through which the signal has passed. Species that use electrical signals for social communication are nocturnal or inhabit murky waters where visual communication is limited. Electrical signals are useful because they are extremely precise; they are limited to use in aquatic environments, though, because air is ineffective as an electrical insulator or conductor.

Comparison of signal types

As we have seen, a wide variety of signals are used in animal communication. Of course, each has its advantages and disadvantages, and are more useful in certain situations than in others. Otherwise, evolution would have only produced one type. In, we can see a comparison of visual, acoustic, chemical, and tactile signals. Acoustic and chemical signals are useful when obstacles stand between

the signaler and the receiver, whole tactile and visual signals are not useful unless there is a clear path. Chemical signals can persist for long periods of time, while other signal types occur in real time, and so are only fleeting messages.

	Visual	Acoustic	Chemical	Tactile
Range	Good	Very Good	Very Good	Poor
Ease of locating source	Very Good	Good	Varies	Very Good
Going around obstacles	Poor	Very Good	Very Good	Poor
Speed	Very Good	Very Good	Poor	Very Good
Persistence	Poor	Poor	Very Good	Poor

Figure %: Comparison of Signal Types

Communication and ecology

The analysis of animal communication is directed at discovering its functions, broadly characterised earlier in this article. Variations in modalities used, and in mapping relationships between signals produced and responses elicited by them, should in theory be explicable in terms of the details of the environmental circumstances a given species is adapted to, even though this is only supported by case-by-case examination. The physical environment clearly limits choices of modality in the example of the absence of visual communication in nocturnal species, and more detailed inspection reveals correspondences between, for instance, the precise characteristics of vocalizations and those most appropriate for optimal dispersion in given geographical conditions. The social environment of a species and individuals within it is no less important. For instance in the example of Bonelli's warbler, above, the fact that transposing its song upwards in pitch slightly severely reduced responses to it, while transformation downwards had little effect, is almost certainly because this species often co-exists with another which has a similar but higher-pitched song. More general comparisons suggest that both song-birds and forest primates which need to communicate acoustically over relatively short distances, with comparatively little "noise" from other similar species, have a larger species repertoire of calls with greater amounts of individual variation, than those in which group or species members are more widely dispersed, with greater likelihood of between-species confusions.

Inherited constraints and universals

The assumption that animal communication systems have evolved to fit particular species circumstances leads, other things being equal, to the expectation that both the structure of the motor and sensory organs employed for these purposes, and the behaviour-controlling functions of the nervous systems that control them, will be innately determined. Biologists are not surprised by species-specific universals. There are however exceptions to this expectation, at least in birds and mammals. The main category is that individuals in social groups learn to recognise each other by sight, sound and smell. Learning to produce communicative signals as opposed to perceptual learning is less widespread, but is a significant factor in bird-song. Vocal learning of one sort or another is pervasive in birds and ubiquitous, though not universal, in song birds, for reasons which remain obscure, but which may include, in varying species:

i) Evolutionary factors which favour the establishment of local dialects;
ii) Benefits to the individual of increased variability of vocal production, either because of distinctiveness for recognition or where degree of elaboration of song influences female mate choice and/or impresses other males.

An important distinction within avian vocal learning is that between within-species imitation (and creative elaborations within a specific species pattern), and vocal mimicry, where birds such as the Mockingbird or Starling adopt recognizable parts of other species' calls. For within-species imitation, conformity to a standard form may reflect in some cases social proximity in early learning (hand reared parents may transmit human sounds learned by imitation to their offspring) but experimental evidence for others has led to the concept of an "auditory template", which enables the young to identify their own species' song as what should be modelled and elaborated. Inter-species mimicry, on the other hand, demonstrates, (in approximately 15% of all species), a part of the language learning device in birds which is more genuinely a *tabula rasa*. Its existence remains an evolutionary puzzle, since it occurs in a wide range of habitats and families, although it is more common in non-migratory insectivorous species. However, not surprisingly, the only common factor is that it occurs in birds that are dependent on vocal learning for their own species' calls, and mimicry may be an extreme case of the usefulness of variability, as well as serving special cases such as the mimicry of host by parasite species, and the recording of local or migratory histories.

The evolution of human language

Little has been learned about the evolution of human language from the study of animal communication. Marginally more useful comparisons may be made possible however, if human language is no longer either a) held to be entirely culturally determined, or, b), assumed to be substantially and crucially innate, but for reasons that are fortuitous and non-Darwinian. The case has recently been made that innate capacities underlying human language must have evolved by Darwinian processes (Pinker and Bloom 1990), and accepting this implies that there may be points of commonality between the evolutionary biology of human language and animal communication systems, even though there are striking differences in the nature of the end-products.

Viewed from the stand-point of natural history, human language remains unique in its syntactic and propositional character, and the degree to which it supports cultural transmission and change. It is also unusual in that its functional importance is not matched by noticeable evolution of the structure of the peripheral sensory and motor mechanisms which support it, possibly because it is an extreme case of a system for a large and highly variable repertoire of low-energy signals transmitted within groups of individuals at close quarters, rather than one for broadcasting a fixed set of messages reliably at long distances. It is not remarkable in having both innate and acquired aspects, but far exceeds any other natural system in the degree to which sociocultural factors are superficially predominant. Despite features which are arguably unique products of evolutionary processes, and many others which clearly post-date biological changes (for example historical language development, and in particular all aspects of language that are dependent on its *written* forms) human language continues to serve some of the same functions as animal communication systems - identification of the individual, recognition of gender and group, and the other social functions of greeting, affiliation, assertion and attachment.

Probable questions:

1. What do you mean by communication?
2. Discuss in details about different types of communication process.
3. Discuss the functions of communication.
4. Discuss different types of channels of communication.
5. Briefly discuss about visual communication.
6. Briefly discuss about acoustic communication.
7. What are the importance of communication?
8. Write short notes on origin of signals.
9. Discuss on the mechanism of receiving Visual Signals.
10. Elaborate the relationship between communication and ecology.

Suggested Readings:

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Unit VI

Evolution of feeding behaviour: optimal foraging theory

Objective: In this unit you will know about evolution of feeding behaviour and optimal foraging theory.

Introduction

Foraging Behaviour:

Expression of all behaviour in all animals requires energy. Animals are essentially heterotrophs and food is prime source of energy in animals. Food may be gained in various ways, all of which are together called "Foraging". This behaviour are collectively called Foraging Behaviour.

On the basis of range of food item animals consume, they can be grouped into 2. Those primarily feed exclusively on only one kind of food are called "Specialized", some ant ate only Spider's egg, in contrast those taking many kind of food are called generalized.

Food finding ability of one species may depend on foraging strategy of another species. Foraging investment of one species may be parasitized by another species Lapwing *Vanelus* sp. feeds on earth worm which are also favourite food for Black Headed Gulls. Gulls often undertake Arial chasing of Lapwing to catch whole of Earthworm but is often expensive. Thus a Gull often attack a Lapwing just when it obtains a prey.

Selective Food:

After finding potential food, a forager has to decide which to attack and which to ignore for its own benefit. The first factor that influences a consumer decision in selecting food is Palatability of alternate food. Both herbivore and carnivore foragers select good and less edible food on the basis of potential toxicity. Plant eating animals like leaf cutting ant (*Atta cephalotes*) or Passel-eared squirrel select plenty as their food item, which contain low amount of terpinoids. Terpinoides are toxic substances, which plants incorporate in their leaves to repel consumers. Similarly, a bat chooses a frog rather than a poisonous toad through their calls though. Later is more abundant in locality.

Optimal Foraging Theory:

Introduction

Optimal foraging theory a theory, first formulated in 1966 by R. H. MacArthur and E. R. Pianka, stating that natural selection favours animals whose behavioural strategies maximize their net energy intake per unit time spent foraging. Such time includes both searching for prey and handling (i.e. killing and eating) it. The theory was originally devised in an attempt to explain why, out of the wide range of foods available, animals often restrict themselves to a few preferred types. The prediction is that an animal strikes a balance between two contrasting strategies: spending a long time (i.e. using more energy) searching for highly `profitable' food items, or devoting minimal time (i.e. using less energy) to more common but less profitable food items. Various factors can cause animals to deviate from optimal foraging. For example, the risk of predation may force the animal to select less profitable food items in a relatively safe location, rather than opting for the energetically most efficient feeding strategy.

The absolute limits of the range of food types eaten by a consumer in a given habitat are defined by morphological constraints, but very few animals actually eat all of the different food types they are capable of consuming. Optimal foraging theory helps biologists understand the factors determining a consumer's operational range of food types, or diet width. At the one extreme, animals employing a generalist strategy tend to have broad diets; they chase and eat many of the prey/food items with which they come into contact. At the other extreme, those with a specialist strategy have narrow diets and ignore many of the prey items they come across, searching preferentially for a few specific types of food. In general, animals exhibit strategies ranging across a continuum between these two extremes.

When food differs in size but not in toxicity selecting a larger prey is more profitable because it is likely to contain more calorie. Optimal Foraging is forwarded in the analysis of such foraging behaviour. Optimal theory predicts optimal solution to an ecological problem and optimal solution is that which raise the fitness of an individual more than other alternatives (Fig 1).

Many birds take mussels as their food, Oyster catcher (*Haematopus* sp.) is such a species the mussels larger in size (Greater than 50 mm in length) provide more calorie. Therefore birds should prefer large mussels as their food. Observation support that bird select larger than average sized mussel (> 35 mm and < 45 mm) not really the large one. To solve this mystery it has been found that larger mussels are difficult to break by catchers so it is not beneficial to handle. A very large prey though it is profitable apparently. Again most large sized mussels are covered by barnacles; each additionally makes them tougher to break open. Thus considering the factor-

1. Calorie richness and
2. Time wasted to open the mussel, bird select > 30 mm to < 45 mm size in a realistic view.

Not only calorie richness, food selection may also be influenced by predation risk many animals which are easy prey to their predators, are extremely reluctant to travel far from their retreats though there are vast food resources nearby. Eg: *Marmota* sp. are small herbivore mammals. Juvenile marmots are involved and less experienced and are easy prey of eagles. They travel a short distance from the burrows than the adults do and spend more time to look around for approaching predators, than they spend for foraging.

Retozacs made several observation while watching foraging crows.

1. Crows picked up only large Whealks about 3.5 to 4.4 cm long.
2. They flewed up about 5 m to drop their choosen Whealks
3. They kept trying until the Whealks brone, even if more fling were required.

Zacs sound to explain the close behaviour by determining whether the birds' decision was optimal in terms of maintaining whelks available for consumption, per unit of time spend foraging. The optimal hypothesis yield following prediction.

1. Large Whelks should be more likely than small one to shutter after a drop of 5m.
2. Drops of less than 5m should yield a reduced breakage rate whereas drop of much more than 5m should not greatly improve the chance of opening the Whelks. The probability that a Whelks will break should be independent of the number of times, it has already been dropped.

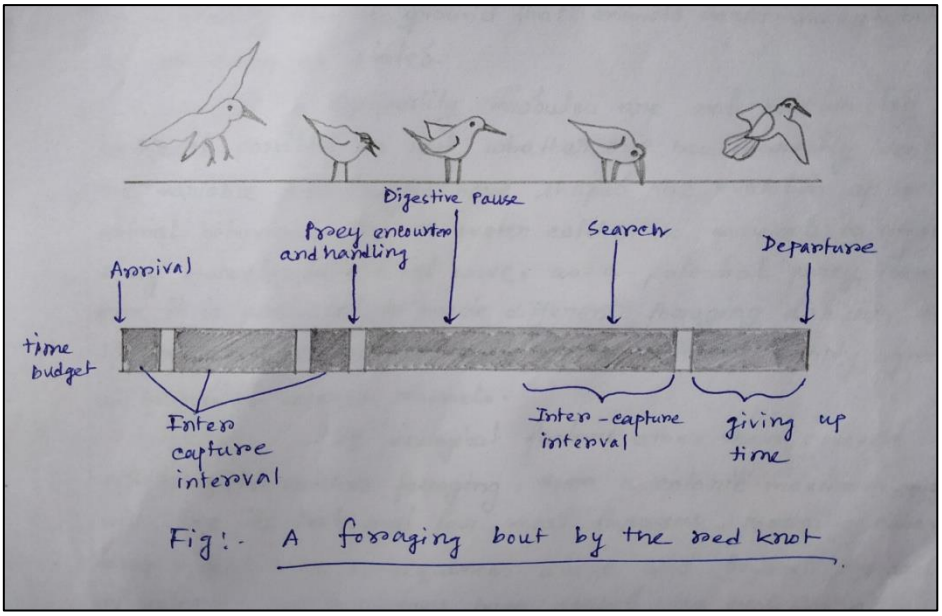


Fig 1

Importance

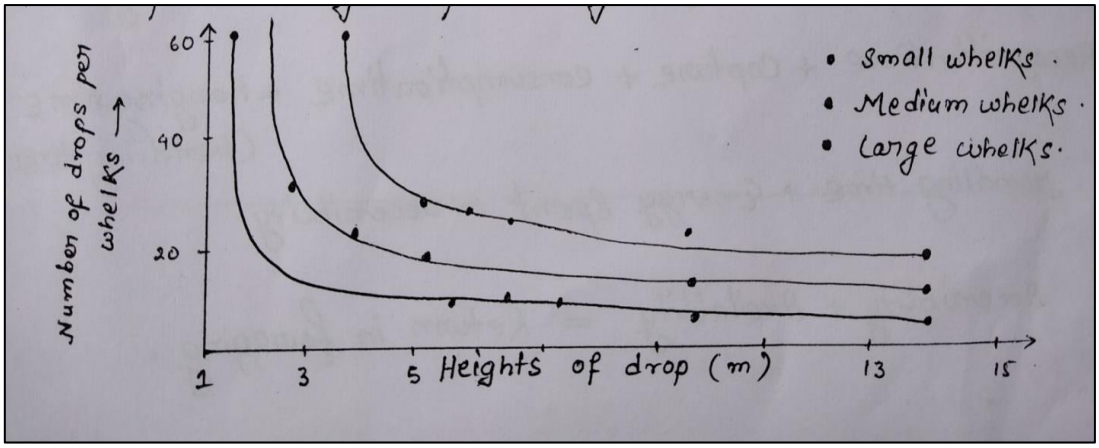
Foraging is critical to the survival of every animal. More successful foragers are assumed to increase their reproductive fitness, passing their genes on into the next generation.

Criticism of Optimal Foraging Theory:

North-western coast and European oyster catchers choose prey that provide a maximum calories in relation to time spent in foraging. But some birds have been under controversy, and some researchers have criticized the use of optimality on the ground that animals do not always behave as efficiently as stated.

Optimality modules are not constructed to make it possible to test whether one has correctly identified the variable that might have shaped the evolution of an animal behaviour. If an oyster catcher is assumed to consider every consider every mussels in a tidal wave, as a potential prey item than it is predicted to made different foraging decision than if the modular assumes that oyster catchers simply ignore all barnacles covered mussels.

If ecological factors other than calorie intake effect oyster catcher foraging, then a calorie- maximum model will take its test and for most foragers, foraging behaviour does indeed have consequences above and beyond the acquisition of calorie. The predators have shaped the evolution of an animal's foraging behaviour, then the kind of optimality model you might choose to construct and test could not focus solely on calorie gain vs calorie expenditure. If foraging express an animal to risk of sudden death, then when the risk is high we could expect foragers to sacrifice short term choice of calorie gain for long term survival.



Foraging efficiency is a matter of trade-off between different alternative

Some species spend much energy while foraging but relatively little time. On contrary other species have low foraging energy expenditure but lot of time in foraging. The rate at which energy is spent, depend upon availability and accessibility of food. In fact foraging efficiency is a matter of trial between priorities. They may include energy gain vs energy expenditure or energy gain vs losing to rivals etc.

In foraging animals, it can be compared with shopping in a supermarket. First and foremost limit of a shopper is the availability of money equivalent to amount energy spent in foraging. Second limitation is amount of time available to select on item. In foraging animal this is equivalent to hanging time required to recognize, capture and process the food item. Finding a prey spend upon its availability. If available, an animal need some time to recognize it. If prey captured successfully, animal take some time to consume it, therefore {Hanging time= (recognition+capture+consumption time)}

Hanging time plus energy of an animal spent to obtain its prey may have accessibility. The returns of an animal obtained per unit time spent in foraging depend on availability and accessibility of the resource.

- Density of prey in environmental= availability.
- Recognition time+capture+consumption time= hanging time or handling time.
- Hanging time + energy spent= accessibility
- Accessibility+ availability= return in foraging

When the prey is profitable for a predator, can be calculated. The net energy value of a prey item is determined by the substitution of energy spent in handling and digestion of prey from gross energy value of the prey.

Net energy/ handling time= profitability.

Animals having a nest or colony, may take capture to home. This type of foraging is called central based foraging and the (distance between central place and foraging area= travel distance)

Net energy value of prey equivalent to-

(Gross energy values- energy spent in handling and digesting).

Optimal foraging theory and its relevance in ecology

Theory:

Behavioural ecology deals with the evolutionary mechanisms for animal behaviour due to pressure from the ecological system. This investigates how the behaviour of an organism is related to the ecosystem. In the past, ecologists took little importance to study the behaviour of organism, but later the studies on survival and reproduction made the behavioural studies more important in ecology. An organism performs so many things such as food searching, defence mechanisms, mate location etc. for their growth and survival. The habitat and the food on which the organism depends are critical factors to study the behaviour of the organism. Most of the research on behavioural ecology looks at the problems of the organisms such as obtaining its food for survival, avoiding being eaten and reproductive mechanisms. Foraging defines the searching of food and exploiting the food resources by the organism. Foraging theory is a branch of behavioural ecology that deals with the foraging behaviour of the organisms with respect to the environment where the organism lives. Optimal foraging is a field in which biologists have used optimization theory to make quantitative predictions about the feeding behaviour which can be then be tested by observation and experiment. The assumption of optimal foraging theory is that the individuals will be energy maximizers or time minimizers. Energy maximizers try to find most energy from the ecosystem while the time minimizers try to get energy in a least time. Since the energy is a limiting factor, such an approach is useful to study the organism's behaviour in the ecosystem. Optimal foraging theory was first proposed by Robert MacArthur, J M Emlen, and Eric Pianka in 1966. Optimal foraging illustrates the organisms forage in such a way as to maximize their net energy intake per unit time. The first assumption of the optimal foraging theory is natural selection will only favour behaviour that maximizes energy return. Understanding the rules that shape the foraging behaviour of individuals in the ecosystem has been a central focus of behavioural ecology for the last decades.

Optimal Foraging

Optimal foraging theory defines the nature of the organisms forage in such a way to maximize the net energy intake of the organism per unit time. The organism behaves in such a way to consume food having most calories with in a least time period. Optimal foraging theory uses predators for the analysis. Predators are categories into two searching and sit-and-wait. A searching predator moves throughout its habitat and find its prey. That means actively foraging predators are characterized by their frequent wandering movements. A sit-and-wait predator waits for its prey to near its point of observation. That is some predators attack their prey from ambush, whereas others usually hunt while on the move. A sit-and-wait strategy is mostly relying on moving preys or high prey mobility and the prey density must be relatively high. In order to favour the sit-and-wait tactic, predator's energy requirements must be low. Whereas searching predators encounter and consume non-moving types of prey population. The success of foraging pattern of 'searchers' is influenced by the prey density and prey mobility along with the predator's energetic requirements. Generally it should be higher than those of sit-and-wait predators. However the searching abilities of the predator and the spatial distribution of its prey are paramount. The sit-and-wait foraging mode is less common during periods of prey scarcity than the widely-foraging pattern. Some common examples of ambush predators include snakes, fish and other reptiles such as crocodiles as well as birds, some mammals and spiders.

Case Studies on Sit and Wait Predator

- **Foraging of Kingfisher**

Kingfisher is a sit-and-wait predator (Fig 2) whose optimal foraging strategy is to maximize the energy that it gains during each foraging course. It usually waits for prey to come within a striking distance. It waits in one place for long periods of time and makes decisions regarding when to hunt or when not to hunt the prey that it sees. A major part of this decision depends on how far the prey is from its predator. The Common Kingfisher hunts from a perch above the water, on a branch, beak pointing down as it seeks for prey. When food is detected, it dives steeply down to grab its prey. For example, consider a kingfisher waiting on a perch on a branch, looking down at a river and choosing which fish to go for. For the sake of understanding, let us assume that the pattern of the foraging area as a semicircle around it and the size and behaviour of all the fishes are same. When a kingfisher takes decision to grab a particular fish, it dives from its perch, seizes the fish, and comeback to its perch.

Fig 2: Kingfisher, a sit-and-wait predator

- **Optimal foraging in crows**

Crows (*Corvus caurinus*) in coastal area of Canada feed on shellfish (Fig 3). They hunt for whelks (*Thais lamellosa*) at low tide on the west coast. Having found a whelk, they fly with it in their beak to above a nearby rock.

Fig 3: Flying Crow; natural scavenger

They stall and drop the whelk from the air hence to smash its shell on the rock thereby exposing the flesh inside it. A crow has to drop each whelk several times to break the whelk open.

A crow has to expand its energy to fly upwards. The crows might drop whelks from a height at which the crows would minimize the total upward vertical flight required per whelk eaten. If whelks are dropped from near the ground, many drops are required to break open the shell.

Zach calculated the dropping height that minimizes the total upward vertical height is close to average of 5.2 m. Zach also suggested that greater the height from which a whelk is dropped, the more the chance that the whelk will fragment into small fragments, among those some are too small to retrieve. This may be the reason why the crows usually fly to a height of about 5 to 5.5 m rather than above 10m.

Factors affecting the foraging behaviour

1. The energy spent in waiting and pursuit of the prey depends on the size of the foraging area. Increasing the size of the foraging area decreases the time and energy spent in waiting for a fish (prey) to come into sight as there is a chance of choosing more fishes.
2. Increasing the size of the foraging area increases the average time and energy spent in capturing the prey, because the kingfisher has to fly longer distance to catch its prey.
3. The area of the foraging system determines the abundance of the prey in that ecosystem(More prey in the maximum foraging area) In case of Kingfisher, this means that increasing the size of the foraging area decreases the waiting time of the kingfisher for fish to appear. Waiting time is then proportional to the reciprocal of the total abundance.

Probable questions:

1. What do you mean by foraging behaviour?
2. What do you mean by selective food?
3. Discuss briefly the optimal foraging theory.
4. Discuss optimal foraging theory and its relevance in ecology.
5. Discuss the factors which affects the foraging behaviour.
6. Write down the foraging behaviour of Kingfisher.

Suggested Readings:

1. Alcock, J. (2001). Animal Behaviour: An Evolutionary Approach. , Sinauer Associate Inc., USA.
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HARD CORE THEORY PAPER (ZHT- 411)

Animal Behaviour and Vector Biology

Group B: Vector Biology

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZHT-411 (Vector Biology)	**VII**	Life cycle, mode of transmission, control and importance of *Anopheles* sp., *Culex* sp., *Aedes* sp	**1.5**	**1**	**1**	**85-110**
	VIII	Life cycle, mode of transmission, control and importance of Ticks and mites		**1**	**1**	**111-133**
	IX	Life cycle, mode of transmission, control and importance of Sand flies		**1**	**1**	**134-143**
	X	Life cycle, mode of transmission, control and importance of Tabanid fly		**1**	**1**	**144-151**
	XI	Life cycle, mode of transmission, control and importance of Black fly		**1**	**1**	**152-160**
	XII	Life cycle, mode of transmission, control and importance of Flea		**1**	**1**	**161-169**

Unit VII

Life cycle, mode of transmission, control and importance of *Anopheles* sp., *Culex* sp., *Aedes* sp

Objective: In this unit you will know about Life cycle, mode of transmission, control and importance of *Anopheles* sp., *Culex* sp., *Aedes* sp

Introduction

Anopheles is a genus of mosquito first described and named by J. W. Meigen in 1818. The *Anopheles* mosquito is known universally as the Malaria Mosquito species because it is considered the primary vector of the disease. It is also considered a transmitter of heartworm in dogs. About 460 species are recognised; while over 100 can transmit human malaria, only 30–40 commonly transmit parasites of the genus *Plasmodium*, which cause malaria in humans in endemic areas. When resting, the stomach area of the Anopheles mosquito points upward, rather than being even with the surrounding surface like most mosquitoes. *Anopheles gambiae* is one of the best known, because of its predominant role in the transmission of the most dangerous malaria parasite species (to humans) – *Plasmodium falciparum.*

The life cycle of *Anopheles* sp.

Life cycle of *Anopheles* mosquito is discussed below

Copulation:

Mosquitoes copulate while flying during the night. It is believed that the pitch of sound produced during flight is higher in females, and this helps the male mosquitoes to locate the female mosquitoes and copulate. The female Anopheles mosquito will mate several times in her short lifespan, producing eggs after she has found a blood meal. Although she only lives a few weeks to a month at most, she will have been able to produce thousands of eggs during that time.

Oviposition:

After copulation the female Anopheles lays about 40 to 100 after midnight in standing water of some pond, ditch, pool, puddle, lake, well, water-storage tanks etc., or even in water containers in our houses. A blood-meal by the female is necessary before oviposition. Female *Anopheles* lays its eggs singly.

Like all mosquitoes, anophelines go through four stages in their life cycles: egg, larva, pupa, and imago. The first three stages are aquatic and together last 5–14 days, depending on the species and the ambient temperature. The adult stage is when the female *Anopheles* mosquito acts as malaria vector. The adult females can live up to a month (or more in captivity), but most probably do not live more than two weeks in nature.[

The life cycle of *Anopheles* has following four stages; egg, larva, pupa and adult (Fig 1). The first three stages are aquatic and last 5-14 days, depending on the species and the ambient temperature. The adult stage is when the female *Anopheles* mosquito acts as malaria vector. The adult females can live up to a month (or more in captivity) but most probably do not live more than 1-2 weeks in nature.

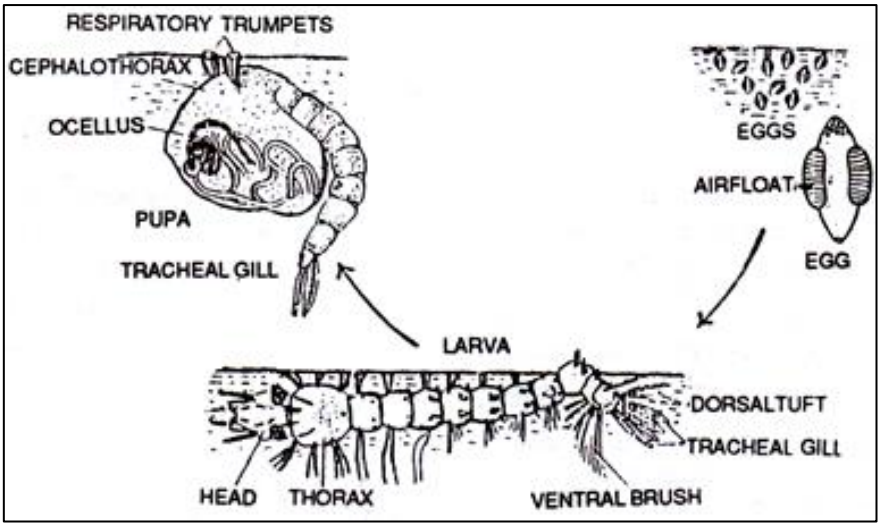


Fig 1: Life cycle of *Anopheles* sp.

The Eggs:

1. The eggs are whitish and boat-shaped.

2. 50 to 80 eggs are laid on water-surface singly and are floating horizontally through air floats.

3. In 24 hrs. they hatch into larvae.

The Larva:

1. The larvae are free swimming.

2. They lie horizontally parallel to the surface of water.

3. Their body is elongated, somewhat cylindrical and is divisible into head, thorax and abdomen.

4. The head bears paired antennae, eyes, feeding brushes and mandibulate mouth parts.

5. Thorax is broad un-segmented and bears tufts of hair for swimming in water.

6. Abdomen is long, 9 segmented and is having palmate hairs.

7. The 8th segment bears a small siphon and paired spiracles on the dorsal surface.

8. Ninth segment bears two pairs of tracheal gills and two tufts.

9. It hatches out into pupa after 4 moultings.

The Pupa:

1. The body of pupa is comma shaped (,) and is covered over with a transparent puparium.

2. In pupa the head and thorax are united into cephalothorax which has become greatly distended and bears paired respiratory siphons, compound eyes, jointed antennae and rudiments of other appendages.

3. The abdomen is long, 9 segmented, curved and flexed completely below cephalothorax and bears palmate hairs and a pair of paddles attached to the 8th segment for swimming.

4. It metamorphoses into adult after a short- free swimming life.

Adult:

The duration from egg to adult varies considerably among species, and is strongly influenced by ambient temperature. Mosquitoes can develop from egg to adult in as little as five days, but it can take 10–14 days in tropical conditions. Like all mosquitoes, adult *Anopheles* species have slender bodies with three sections: head, thorax and abdomen.

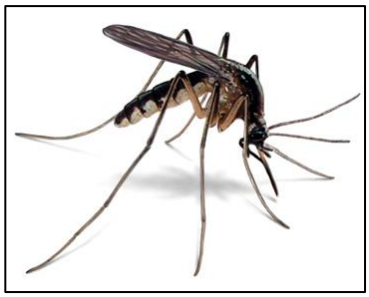


Fig: *Anopheles* sp.

The *head* is specialized for acquiring sensory information and for feeding. It contains the eyes and a pair of long, many-segmented antennae. The antennae are important for detecting host odours, as well as odours of breeding sites where females lay eggs. The head also has an elongated, forward-projecting proboscis used for feeding, and two maxillary palps. These palps also carry the receptors for carbon dioxide, a major attractant for the location of the mosquito's host.

The *thorax* is specialized for locomotion. Three pairs of legs and a pair of wings are attached to the thorax.

The *abdomen* is specialized for food digestion and egg development. This segmented body part expands considerably when a female takes a blood meal. The blood is digested over time, serving as a source of protein for the production of eggs, which gradually fill the abdomen.

Anopheles mosquitoes can be distinguished from other mosquitoes by the palps, which are as long as the proboscis, and by the presence of discrete blocks of black and white scales on the wings. Adults can also be identified by their typical resting position: males and females rest with their abdomens sticking up in the air rather than parallel to the surface on which they are resting.

Adult mosquitoes usually mate within a few days after emerging from the pupal stage. In most species, the males form large swarms, usually around dusk, and the females fly into the swarms to mate. Males live for about a week, feeding on nectar and other sources of sugar. Females will also feed on sugar sources for energy, but usually require a blood meal for the development of eggs. After obtaining a full blood meal, the female will rest for a few days while the blood is digested and eggs are developed. This process depends on the temperature, but usually takes 2–3 days in tropical conditions. Once the eggs are fully developed, the female lays them and resumes host-seeking.

Breeding Habits

The female *Anopheles* mosquito will lay her eggs in a wide range of locations. Malaria mosquito breeding grounds include fresh water or salt-water, vegetative or non-vegetative, shady or sunlit. Ground pools, small streams, irrigated lands, freshwater marshes, forest pools, and any other place with clean, slow-moving water are all considered prime Malaria mosquito breeding grounds for egg-laying.

Females, particularly fertilized females, may survive winter by hibernating in caves, which means the malaria breeding cycle can virtually last year-round in some locations. Eggs are capable of surviving

cold temperatures; however, freezing usually kills eggs. Learn about the malaria mosquito bite, as well as the malaria mosquito species.

Relationship between *Anopheles* sp mosquito and malaria disease

Only certain species of mosquitoes of the *Anopheles* genus—and only females of those species—can transmit malaria. Malaria is caused by a one-celled parasite called *Plasmodium.* Female *Anopheles* mosquitoes pick up the parasite from infected people when they bite to obtain blood needed to nurture their eggs. Inside the mosquito the parasites reproduce and develop. When the mosquito bites again, the parasites contained in the salivary gland are injected and pass into the blood of the person being bitten.

Malaria parasites multiply rapidly in the liver and then in red blood cells of the infected person. One to two weeks after a person is infected the first symptoms of malaria appear: usually fever, headache, chills and vomiting. If not treated promptly with effective medicines, malaria can kill by infecting and destroying red blood cells and by clogging the capillaries that carry blood to the brain or other vital organs.

There are four types of human malaria: *Plasmodium vivax, P. malariae, P. ovale* and *P. falciparum.* are the most common forms. Falciparum malaria—the most deadly type—is most common in sub-Saharan Africa, where it causes more than 400 000 deaths a year.

In recent years, some human cases of malaria have also occurred with *Plasmodium knowlesi* – a species that causes malaria among monkeys and occurs in certain forested areas of South-East Asia.

Malarial parasite (*Plasmodium vivax*)

Plasmodium vivax is a protozoal parasite and a human pathogen. This parasite is the most frequent and widely distributed cause of recurring malaria. Although it is less virulent than *Plasmodium falciparum*, the deadliest of the five human malaria parasites, *P. vivax* malaria infections can lead to severe disease and death, often due to splenomegaly (a pathologically enlarged spleen). *P. vivax* is carried by the female *Anopheles* mosquito; the males do not bite.

In a study by the London School of Hygiene & Tropical Medicine researchers found that female mosquitoes carrying malaria parasites are significantly more attracted to human breath and odours than uninfected mosquitoes. The research team infected laboratory-raised *Anopheles gambiae* mosquitoes with Plasmodium parasites, leaving a control group uninfected. Then tests were run on the two groups to record their attraction to human smells. Female mosquitoes are particularly drawn to foot odours, and one of the tests showed infected mosquitoes landing and biting a prospective host repeatedly. The team speculates that the parasite improves the mosquitoes' sense of smell. It may also reduce its risk aversion.

Systemic Position

Kingdom: Animalia

Phylum: Protozoa

Class: Sporozoa

Order: Haemosporidia

Genus: *Plasmodium*

Species: *Vivax*

Malaria is one of the most widely known diseases since time immemorial. It is caused by a pathogenic protozoan of blood, Plasmodium.

Four species of *Plasmodium*, viz., *P. vivax, P. falciparum, P. malariae* and *P. ovale* are so far known to infect human beings causing different types of malaria. Female *Anopheles* mosquito serves as the carrier or vector hosts and transmits plasmodium from person to person. *Plasmodium* is an intracellular parasite in RBCs of man.

It is also reported from birds, reptiles and various mammals. Plasmodium is widely distributed in tropical and temperate countries the world over. Plasmodium vivax requires two hosts to complete its life cycle- a primary or definite host and a secondary or intermediate host. Such a two host life cycle is digenetic. Intermediate host is female Anopheles. In human body the parasite multiplies asexually while in female anopheles it undergoes a sexual cycle followed by an asexual multiplication called sporogony.

Asexual cycle in man:

The normal adult or trophozoite phase of plasmodium occurs in RBCs of human beings. The parasite first invades the liver cells for asexual multiplication.

The life cycle of plasmodium in man is can be studied under the following heads:

(i) Exoerythrocytic cycle:

When an Anopheles mosquito bites a human to suck blood. Plasmodium is inoculated into human blood in the form of a minute infective stage called Sporozoites (fig 2). The injected sporozoites invade the hepatocyte cells in the liver. In the liver cell, a sporozoite actively feeds on its cytoplasm and grows into a large (about 45 in diameter) and spherical adult like form called cryptozoite.

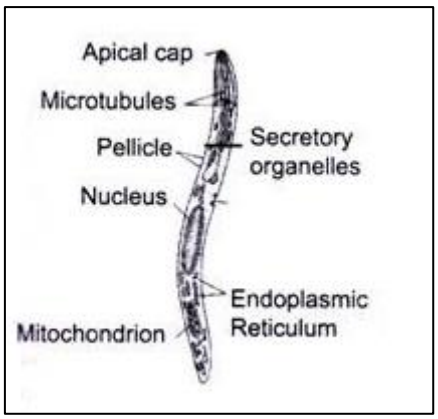


Fig 2: Sporozoite of *P. vivax*

This form multiply into thousands of cryptomerozoites by multiple fission called schizogony (exoerythrocytic schizogony). In such a multiplication repeated nuclear divisions first result into multinucleate organism, and then divides by cytoplasmic segregation around the tiny daughter nuclei. Due to the pressure of cryptomerozoites, the body of cryptozoites as well as the host liver cell ruptures liberating the cryptomerozoites into liver sinusoids. Some of these invade fresh liver cells to continue exo-erythrocytic schizogony, while others remain in blood sream and invade erythrocytes (RBC) to initiate erythrocytic cycle.

(ii) Erythrocyic cycle:

This cycle takes place in RBCs after the RBCs are invaded by cryptomeromerozoites. After invading an erythrocyte, a cryptomeromerozoite soon becomes a rounded, disc like structure called

trophozoites (fig 3). As it grows, a contractile vacuole appears in its centre, pushing the cytoplasm and nucleus to a thin peripheral layer and the parasite attains a ring Ike appearance to represent the signet ring stage.

After some time, the vacuole disappears and the parasite assumes an amoeboid shape. The trophzoites actively feed upon the haemoglobin of RBCs and increases in size till the entire corpuscle gets filled with it. This forms the schizont stage and its cytoplasm contain yellowish-brown pigment granules, the haemozoin. It is formed by the decomposition of haemoglobin. The schizont undergoes asexual multiplication termed as schizogony or merogony.

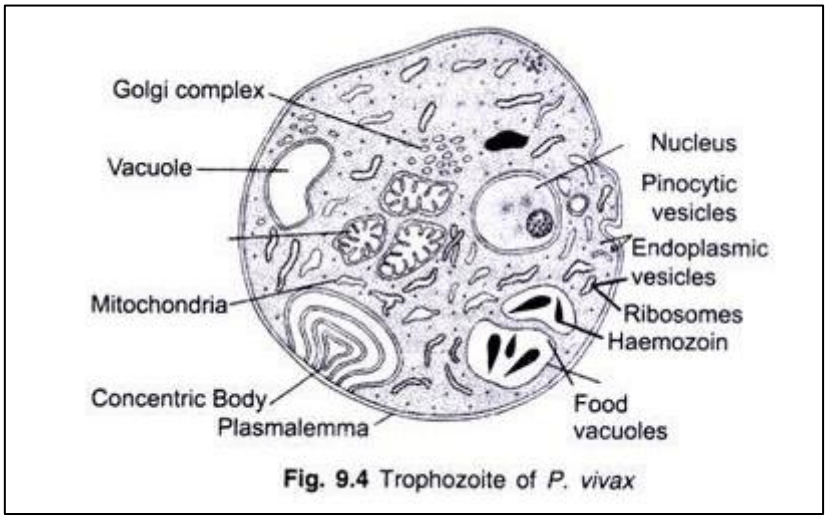


Fig. 9.4 Trophozoite of *P. vivax*

Fig 3: Trophozoite of *P. vivax*

(iii) Schizogony or merogony:

The nucleus or the schizont divides by multiple fission to from 6-24 daughter nuclei which migrate towards the periphery. After some time the totally exhausted erythrocyte bursts liberating the merozoites and the toxic waste (haemozoin granules) into the plasma of blood. These attack the fresh R.B. Cs. And repeat the erythrocytic schozogony. One erythrocytic cycle is completed within 48-72 hours.

As the parasite continues to destroy the R.B.Cs. of the host, the host becomes anemic and its toxin accumulates in the plasma. After about 5 successive erythrocytic cycles the malarial symptoms develop for the first time and the host suffers from paroxysm of chill and fever which are now repeated at the end of each schizogony. Thus the parasite passes a latent period of about 10 to 15 days since its inoculation in the body of host. This period is known as incubation period.

(iv) Formation of gametocytes:

As a result of repeated schizogony in the blood stream, the parasite becomes so potential that its existence is threatened due to lack of fresh R.B.Cs. and the resistance of the host. Consequently, the parasite prepares to enter the new host by the formation of gametocytes. Some of the meozoites, after entering the R.B.Cs. neither form trophozoites nor multiply by binary fission but grow slowly and become compact bodies, the gametocytes. These are of two types:

The more numerous, but small in size and with a large centrally placed nucleus, are the microgametocytes, potentially male. The less numerous but larger in size and with a greater amount of dense cytoplasm and a small nucleus are the macro or mega gametocytes, potentially female. The

mature gametocytes are unable to develop further in the body of primary host and can survive only for two days. They reach the superficial blood vessels and wait for the bite of female Anopheles.

Sexual Life-Cycle in *Anopheles*:

When *Anopheles* sucks the blood of a diseased man, the parasite under different stages of development enters its alimentary canal. But only the gametocytes are able to survive, while others are digested. The gametocytes are set free by the rupture of R.B.Cs. and develop further to form gametes.

(i) Development of male gametes:

The nucleus of microgametocyte divides repeatedly to form 6 to 8 haploid nuclei, as one of these divisions is a reduction division. Each nucleus is surrounded by a little of cytoplasm and metamorphoses into a male gamete. Each has a small body with a nucleus and a cytoplasmic flagellum. By the lashing movement of their flagella the male gametes swim in the stomach fluid.

(ii) Development of female gametes or microgamete's:

The nucleus of the macrogametocyte undergoes reduction divisions forming two nuclei. One of them protrudes out as a polar body and the other comes to lie in a protuberance which is known as reception cone. Thus the macrogamete is formed.

(iii) Syngamy or fertilization:

The actively moving male gamete is attracted by the macrogamete and penetrates it through the reception cone. The nuclei of the two fuses together forming the synkaryon. Syngamy is anisogamous and the zygote thus formed is inert and round.

(iv) Ookinete:

Soon the rounded zygote elongates and assumes the vermiform appearance and becomes motile. It is now known as vermicule or ookinete (fig 4). Its anterior en4 is pointed and with this it penetrates the stomach wall to come to lie in the sub-epithelial tissue underneath the outer limiting membrane. It becomes rounded, secretes a thin membranous cyst and is known as sporont or oocysty. It feeds by absorption and increases in size.

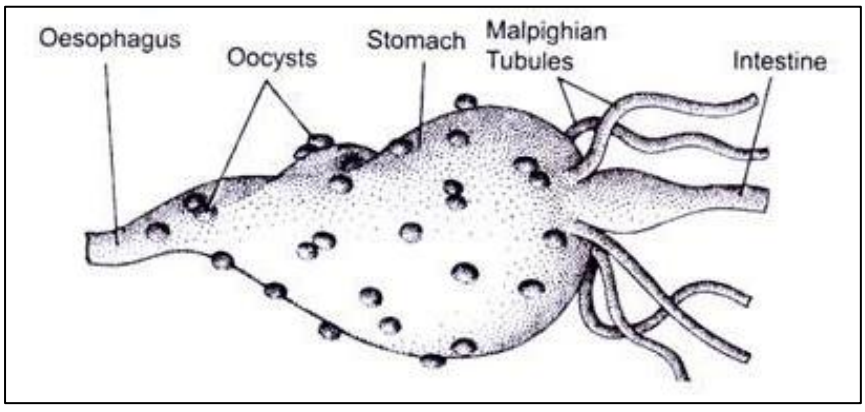


Fig 4: Oocyst in infected mosquito

Sporogony:

The nucleolus of the fully mature oocyst undergoes multiple fission by mitosis producing a large number of daughter nuclei. These get surrounded by fragments of cytoplasm. The irregular uni-

nucleate bodies thus formed are known as sporoblasts. The nucleus in each sporoblast divides repeatedly by mitosis.

The nuclei form spindle-shaped sporozoites. These are liberated in the haemocoel or body cavity by the repture of cyst wall. The sporozoites now move to different body organs and also the salivary gland. With the entrance of parasite in the salivary glands the female Anopheles becomes infective and is able to inoculate the parasite into the blood-stream of healthy persons.

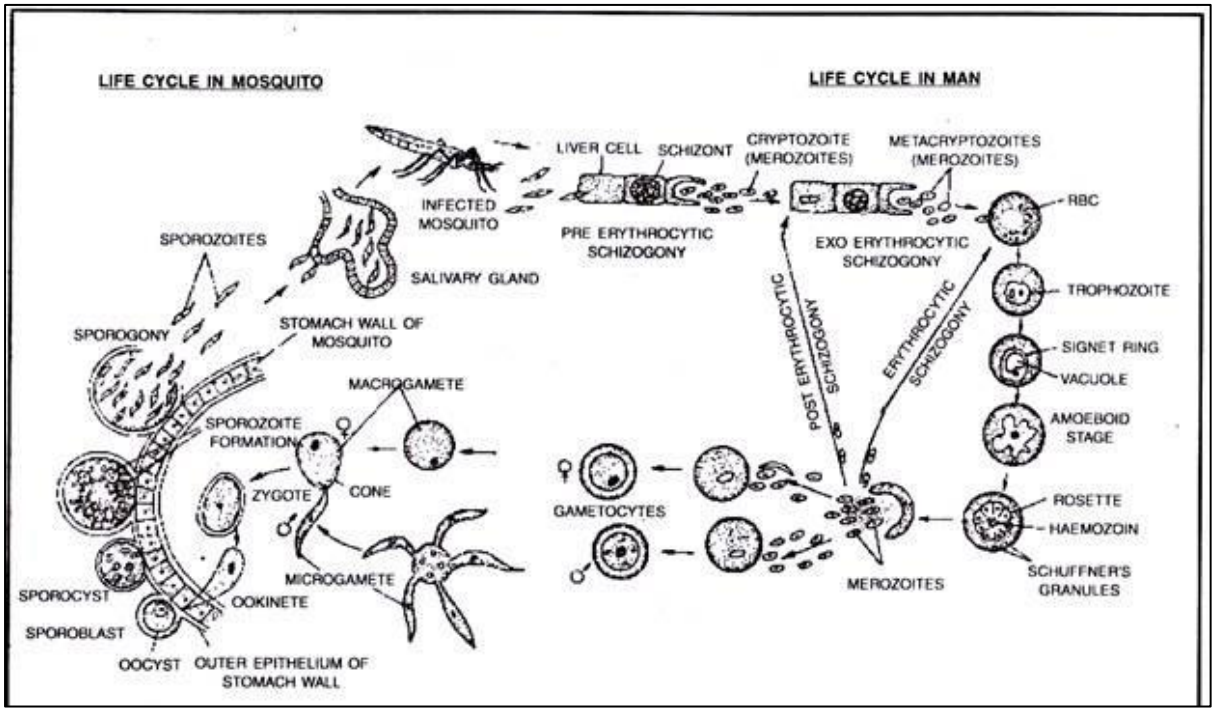


Fig : Life cycle of *Plasmodium* sp.

Mode of transmission of malaria

The female anopheles mosquito is the vector for human malaria. Some 60 species of this mosquito have been identified as vectors for malaria, and their distribution varies from country to country.

The infection is transmitted by the bite of an infected female mosquito – Anopheles. *An. culicifecies* in Rural area & *An. stephensi* in urban area. The mosquito usually bites during dawn & dust time. The mosquito becomes infected by biting a patient with malaria infection. When a mosquito bites an infected individual, it sucks the gametocytes, the sexual forms of the parasite, along with blood. These gametocytes continue the sexual phase of the cycle and the sporozoites fill the salivary glands of the infested mosquito. Once the mosquito becomes infected, it remains so far life. The female mosquitoes can survive upto 4 weeks under normal temperature i.e. 28°C to 30°C and humidity i.e. 60 to 80%. When this female mosquito bites the man for a blood meal, which it needs to nourish its eggs, it inoculates the sporozoites into human blood stream, thus spreading the infection.

Other modes of transmission

Rarely malaria can spread by the inoculation of blood from an infected person to a healthy person. In this type of malaria, asexual forms are directly inoculated into the blood and pre–erythrocytic development of the parasite in the liver does not occur. Therefore, this type of malaria has a shorter incubation period and relapses do not occur.

1. Blood transfusion (Transfusion malaria)

- This is fairly common in endemic areas. Following an attack of malaria, the donor may remain infective for years (1–3 years in *P. falciparum*, 3–4 years in *P. vivax*, and 15–50 years in *P. malariae*).
- Most infections occur in cases of transfusion of blood stored for less than 5 days and it is rare in transfusions of blood stored for more than 2 weeks. Frozen plasma is not known to transmit malaria.
- The clinical features of transfusion malaria occur earlier and any patient who has received a transfusion three months prior to the febrile illness should be suspected to have malaria.
- Donor blood can be tested with indirect fluorescent antibody test or ELISA, and direct examination of the blood for the parasite may not be helpful.
- In endemic areas, it is safe to administer full course of chloroquine to all recipients of blood transfusion.
- In transfusion malaria, pre–erythrocytic schizogony does not occur and hence relapses due to dormant hepatic forms also does not occur. Therefore, treatment with primaquine for 5 (or14) days is not indicated.

2. Mother to the growing fetus (Congenital malaria)

Intrauterine transmission of infection from mother to child is well documented. Placenta becomes heavily infested with the parasites. Congenital malaria is more common in first pregnancy, among non– immune populations.

3. Needle stick injury

Accidental transmission can occur among drug addicts who share syringes and needles.

Culex sp.

Culex is a genus of mosquitoes, several species of which serve as vectors of one or more important diseases of birds, humans, and other animals. The diseases they vector include arbovirus infections such as West Nile virus, Japanese encephalitis, or St. Louis encephalitis, but also filariasis and avian malaria. They occur worldwide except for the extreme northern parts of the temperate zone, and are the most common form of mosquito encountered in some major U.S. cities, such as Los Angeles.

Habit and Habitat

Culex pipiens is found in temperate regions all over the world, and *Culex fatigans* throughout the tropics and sub-tropics. *Culex* lives in houses, in cities and farms, and is abundant also in rural areas. They are most abundant during spring, but hibernate during un-favorable climatic conditions, the adults hide in hollows of trees, caves, crevices, barns, etc.

The life span of male mosquitoes is seldom more than three weeks, they die after fertilizing the females. The females live from four weeks to several months, but they die when all their eggs are laid. *Culex* has several generations in a year.

Life Cycle of *Culex* sp.

The life cycle of *Culex* is discussed here with the help of a diagram. The Life Cycle of *Culex* has following stages: egg, larva, pupa and adult (Fig 5)

The Eggs:

1. They are laid in rafts of about 300 eggs held, together through sticky substance for floating along the surface of water.

2. The eggs are brownish in colour and somewhat oval in shape.

3. They contain air bubbles trapped in the sticky substance to provide buoyancy.

4. They hatch after 24-48 hours into free swimming larvae.

The Larvae:

1. They are the first free swimming stages in the life cycle.

2. The larva lies at an angle to the surface of water in resting condition.

3. The body is elongated and somewhat cylindrical and is divisible into head, thorax and abdomen.

4. The head bears paired antennae, eyes, feeding brushes and mandibulate mouth parts.

5 Thorax is broad, un-segmented and bears tufts of hair for wriggling in water.

6. Abdomen is without palmate hairs.

7. The 8th segment bears a long tubular respiratory siphon at the tip of which lies a spiracle.

8. Ninth segment bears two pairs of tracheal gills and two tufts.

9. It hatches out into pupa after 4 moultings.

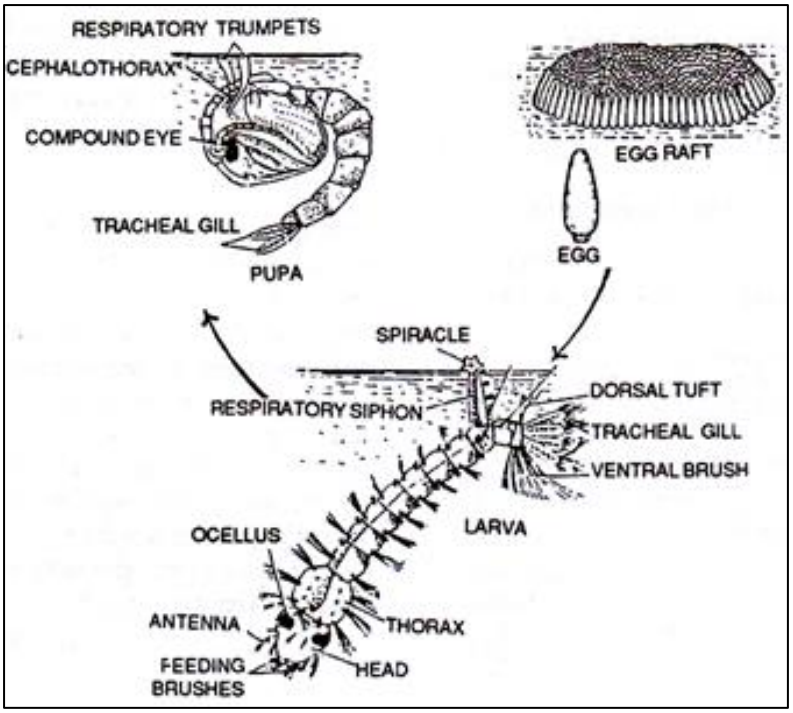


Fig 5: Life cycle of *Culex* sp.

The Pupa:

1. The pupa develops from larva after seven days.

2. The body is comma shaped (,) and is covered over with a transparent puparium.

3. In pupa the head and thorax have united into cephalothorax which has become greatly distended and bears paired short respiratory siphons, compound eyes, long and jointed antennae and rudiments of appendages.

4. The abdomen is long, 9 segmented and curved but not flexed below cephalothorax. It, however, bears a pair of paddles & a pair of tracheal gills attached to ninth segment and palmate hairs on all segments.

5. It metamorphoses into adult after a short free swimming life.

Adult

Depending on the species, the adult *Culex* mosquito may measure from 4–10 mm (0.2–0.4 in). The adult morphology is typical of flies in the suborder Nematocera with the head, thorax, and abdomen clearly defined and the two forewings held horizontally over the abdomen when at rest. As in all Diptera capable of flight, the second pair of wings is reduced and modified into tiny, inconspicuous halteres.

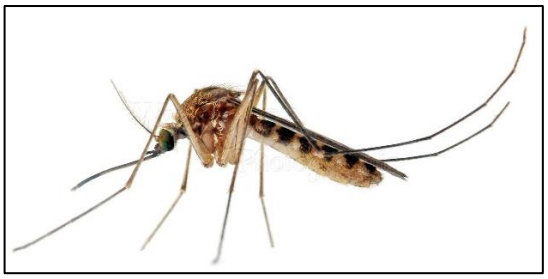


Fig: *Culex* sp.

1. Head:

Head is globular and highly mobile on a slender neck. There are two very large black compound eyes, there are no ocelli. The top of the head has an epicranium below which is a clypeus which is thick and projects in front. There are two filiform antennae, each with 15 joints, the basal segment is the scape which is concealed by a very large globular second segment. The bristles are longer and much more numerous on the antennae of males giving them a bushy appearance. In the female the antennae have rings of few, short bristles, thus, sexes can be distinguished readily by the antennae. The head bears two maxillary palps and a proboscis.

Mouth Parts:

The proboscis is a straight, long tube formed by a fleshy ventral labium which has a deep groove on its upper side, in this groove is a long pointed and ventrally-grooved labrum epipharynx. At the distal end of the labium is a pair of small tactile labella which are reduced labial palps. The groove of the labium also contains five needle-like stylets in a female Culex, they are two mandibles, two maxillae, and a hypo pharynx. The mandibles are finer than the maxillae, but both have saw-like edges on their tips. The hypo pharynx is also needle-like and has a fine salivary duct running through it and opening at the tip, through this duct saliva is poured to prevent coagulation of blood of the victim.

In the male the labrum-epipharynx and the labium are the same as in the female, but the mandibles and maxillae are very short and functionless and the hypo pharynx is fused with the labium.

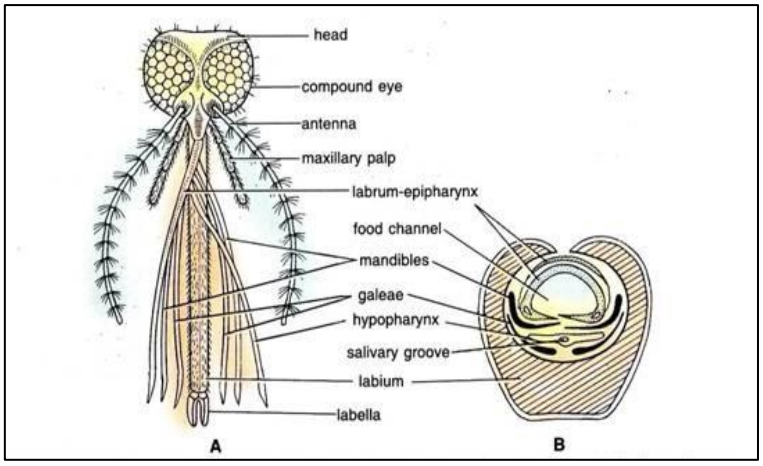


Fig 6: Culex sp. A – Diagrammatic head and mouth parts of a female; B – T.S. of mouth parts

2. Thorax

Thorax is arched, it has mesothorax which is very large and its tergum has three sclerites, a scutum, a trilobed scutellum and a post-scutellum. Prothorax and metathorax are very small. On the thorax there are two pairs of spiracles. From the mesothorax arise a pair of membranous functional wings which are long and narrow.

From the thorax arise three pairs of legs which are very long and slender, they are fragile and have the usual parts of an insect leg, but the coxae are short and tarsi long with five joints ending in a pair of simple claws, below each claw is a pad-like pulvilus. The legs also have many scales and bristles.

3. Abdomen

Abdomen consists of 10 segments of which the first is vestigial and fused to the metathorax; the second to the eighth are clearly seen, each has a pair of spiracles; the ninth and tenth segments are partly telescoped into the eighth. In the female the 10th segment is blunt and bears a pair of cerci, between them is a small post-genital plate which is part of the tenth sternum.

Filariasis

Causes:

The disease filariasis is caused by filarial worms *Wuchereria bancrofti* and *Wuchereria malayi*. They belong to the Phylum – Nemathelminthes and class- Nematoda. The adult worms are slender and thread like in appearance and live in the lymph gland and lympth vessels of man.

The female measures 100 mm in length and 0.25 mm in diameter whereas the males are only 40 mm in length and 0.1mm in diameter. The tail of male is coiled and with two unequal spicules. These two spicules help in the process of copulation. The female is larger than the male (Fig. 7).

Their body colour is creamy white. The anterior end of the parasite is swollen slightly and without lips. Certain species of *culex* mosquito (*Culex fatigans, C. quinquefasciatus*) bring about transmission of the parasites from infected person to healthy persons.

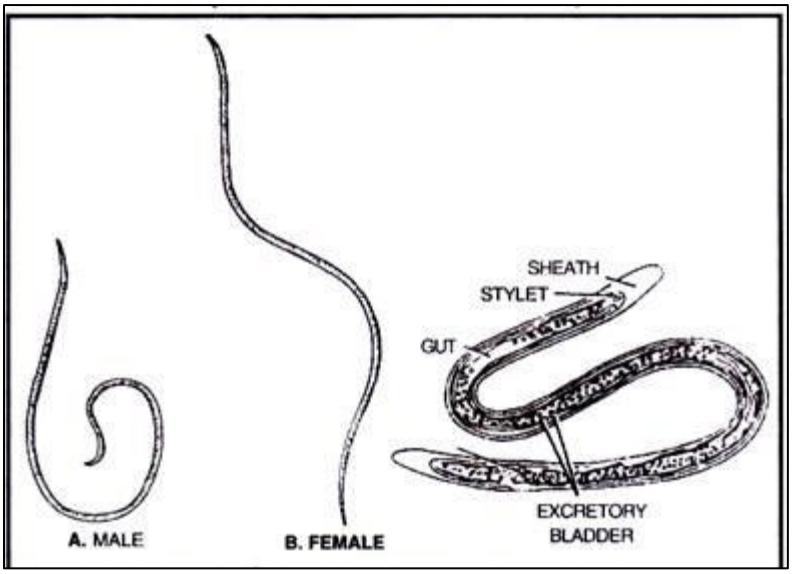


Fig 7. *Wuchereria* sp. A-Detailed structure of male; B-Detailed structure of female and microfilariae of *Wuchereria*

Life Cycle:

Like malarial parasite the life cycle of *Wuchereria bancrofti* is digenetic involving two hosts: man and female *Culex* mosquito (Fig 8). Following mating the female worm lays eggs which hatches to give rise to larvae or juveniles called microfilariae. Each microfilaria is enclosed in a dedicate sheath and measures about 0.22 mm in length. The microfilariae show nocturnal periodicity i.e., they appear in peripheral circulation of man between 10 P.M. and 4 A.M. (to be sucked by mosquito) but live in deeper blood vessels during the day time.

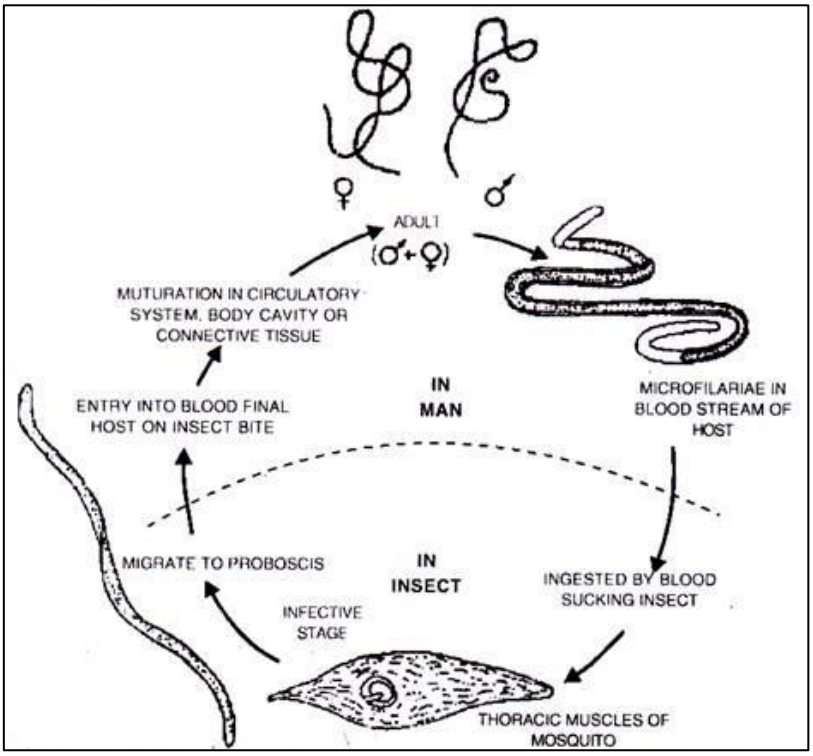


Fig 8: Life cycle of *Wuchereria* sp.

Further development of microfilariae occurs if they are sucked by the mosquito species of *Culex*, *Anopheles* and *Aedes* which act as vectors. In the mosquito gut the microfilariae lose their sheath and migrate to thoracic muscles and undergo two moultings (cast off old skin) there. It is the infective stage which requires two weeks time for development.

Then they migrate to the labium of mosquito when the infected mosquito bites a healthy person the microfilariae get under the skin and later migrate into the lymphatic system of man. There they undergo third and fourth moult and develop into adult. In the host body the adults live up to about 4 to 5 years.

Mode of infection of filarial disease

Filarial disease is usually transmitted to man through mosquito biting. The disease can be accidentally transmitted through blood transfusion, when the donor is infected with microfilariae. The entry of the infective stage, microfilaria in the human body is not through direct inoculation into the blood stream by the mosquito during its blood meal.

Instead, when a mosquito with infective larvae in its proboscis feeds on a person, the larvae get deposited usually in pairs on the skin near the puncture site. The larvae then enter through the wound or puncture. Subsequently they reach the lymphatic channels and settle down at some spots like scrotal sac or inguinal lymph nodes or abdominal lymphatics and begin to develop into an adult worm within a period of 5-18 months.

Symptoms of filarial disease

i. Filarial disease is accompanied with fever and headache.

ii. Owing to virulent filarial infection, the microfilariae block the passage of lymph-gland and as a result lymph cannot go back to the circulatory system, causes tremendous unequal swelling of scrotum, legs, mammary glands etc. This condition of swelling is known as elephantiasis.

iii. The skin of the affected region becomes rough and fissured.

iv. In extreme cases, the connective tissue of the affected parts becomes abnormal when the condition becomes further complicated.

Prevention of filarial disease

The filarial disease could be prevented by adopting several prophylactic measures:

i. Eradication of the vector mosquito by using allethrin or other insecticides.

ii. Destruction of mosquito larvae in the breeding ground can be done by using various larvae ides.

iii. Sterilization of male mosquito .should be done by artificial means.

iv. Protection against mosquito bite by using various devices like mosquito-net, mosquito repellent mat or cream etc.

v. As a measure of biological control mosquito larvae eating fishes like Gambusia. Gold fish etc. may be cultured in the breeding habit of mosquito.

vi. Reducing the rate of infection amongst hosts by preventing them from biting the infected individuals.

Treatment of filarial disease

i. No proper or satisfactory treatment is yet known.

ii. Ivermectin (Mectizan) is very effective against the microfilariae, a single dose is being able to reduce skin micro-filarial counts to undetectable levels within seven days.

iii. Treatment of filaria patient with proper drugs, viz. Mel. W (an arsenical preparation) on adult worms has given encouraging result; Hetrazan compounds on micro-filarial worm and paramelaminyl phenyl stibonate on infective larva and immature adult worm etc. that are used to make the blood free of microfilariae.

iv. Edematous limbs are sometimes successfully treated by applying pressure bandages, which force the lymph out of the swollen area of filarial patient.

v. Surgical removal of elephantoid tissue is often possible.

Aedes

Aedes is a genus of mosquitoes originally found in tropical and subtropical zones, but now found on all continents except Antarctica. Some species have been spread by human activity: *Aedes albopictus*, a particularly invasive species, was recently spread to the New World, including the United States, by the used-tire trade. First described and named by German entomologist Johann Wilhelm Meigen in 1818, the generic name comes from the Ancient Greek ἀηδής, aēdēs, meaning "unpleasant" or "odious". The type species for *Aedes* is *Aedes cinereus*.

Some species of this genus transmit serious diseases, including dengue fever, yellow fever, the Zika virus, and chikungunya.

Life Cycle of *Aedes aegypti*

Aedes aegypti is a so-called holometabolous insect. This means that the insects goes through a complete metamorphosis with an egg, larvae, pupae, and adult stage. The adult life span can range from two weeks to a month depending on environmental conditions. The life cycle of *Aedes aegypti* can be completed within one-and-a-half to three weeks (Fig 9)

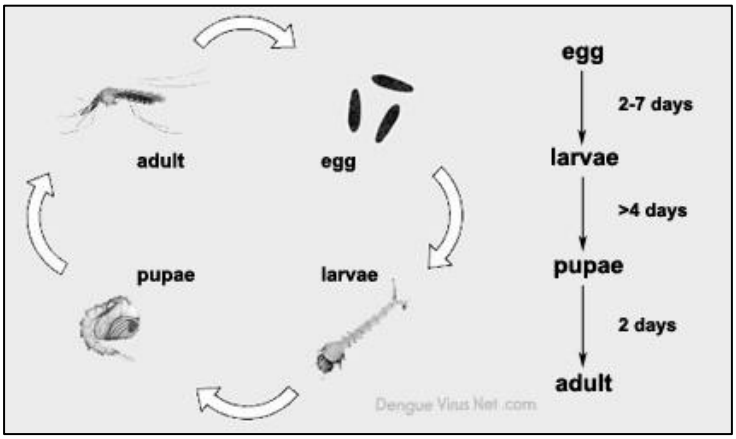


Fig 9: Life cycle of *Aedes aegypti*: there is an aquatic phase (larvae, pupae) and a terrestrial phase (eggs, adults)

Egg

After taking a blood meal, female *Aedes aegypti* mosquitos produce on average 100 to 200 eggs per batch. The females can produce up to five batches of eggs during a lifetime. The number of eggs is dependent on the size of the blood meal. Eggs are laid on damp surfaces in areas likely to temporarily flood, such as tree holes and man-made containers like barrels, drums, jars, pots, buckets, flower vases, plant saucers, tanks, discarded bottles, tins, tyres, water cooler, etc. and a lot more places where rain-water collects or is stored. The female *Aedes aegypti* lays her eggs separately unlike most species. Not all eggs are laid at once, but they can be spread out over hours or days, depending on the availability of suitable substrates. Eggs will most often be placed at varying distances above the water line. The female mosquito will not lay the entire clutch at a single site, but rather spread out the eggs over several sites.

The eggs of *Aedes aegypti* are smooth, long, ovoid shaped, and roughly 1mm long. When first laid, eggs appear white but within minutes turn a shiny black. In warm climates eggs may develop in as little as two days, whereas in cooler temperate climates, development can take up to a week. Laid eggs can survive for very long periods in a dry state, often for more than a year. However, they hatch immediately once submerged in water. This makes the control of the dengue virus mosquito very difficult.

Larvae

After hatching of the eggs, the larvae (fig 10) feed on organic particulate matter in the water, such as algae and other microscopic organisms. Most of the larval stage is spent at the water's surface, although they will swim to the bottom of the container if disturbed or when feeding. Larvae are often found around the home in puddles, tires, or within any object holding water. Larval development is temperature dependent. The larvae pass through four instars, spending a short amount of time in the first three, and up to three days in the fourth instar. Fourth instar larvae are approximately eight millimeters long. Males develop faster than females, so males generally pupate earlier. If temperatures are cool, *Aedes aegypti* can remain in the larval stage for months so long as the water supply is sufficient.

Fig 10: *Aedes aegypti* larvae stage

Pupae

After the fourth instar, the larvae enters the pupal stage (fig 11). Mosquito pupae are mobile and respond to stimuli. Pupae do not feed and take approximately two days to develop. Adults emerge by ingesting air to expand the abdomen thus splitting open the pupal case and emerge head first.

Fig 11: *Aedes aegypti* pupae stage

Adult

Adult *Aedes* mosquitoes are distinguished from other types of mosquitoes by their narrow and typically black body, unique patterns of light and dark scales on the abdomen and thorax, and alternating light and dark bands on the legs. Females are further distinguished by the shape of the abdomen, which usually comes to a point at its tip, and by their maxillary palps (sensory structures associated with the mouthparts), which are shorter than the proboscis. *Aedes* mosquitoes characteristically hold their bodies low and parallel to the ground with the proboscis angled downward when landed

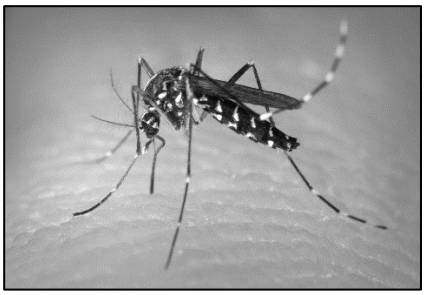


Fig: *Aedes* sp.

Role in disease transmission

Members of the genus *Aedes* are known vectors for numerous viral infections. The two most prominent species that transmit viruses are *A. aegypti* and *A. albopictus*, which transmit the viruses that cause dengue fever, yellow fever, West Nile fever, chikungunya, eastern equine encephalitis, and Zika virus, along with many other, less notable diseases. Infections with these viruses are typically accompanied by a fever, and in some cases, encephalitis, which can lead to death. A vaccine to provide protection from yellow fever exists, and measures to prevent mosquito bites include insecticides such as DDT, mosquito traps, insect repellents, and mosquito nets.

Dengue virus transmission

Dengue viruses are transmitted to humans through the bites of infective female *Aedes* mosquitoes. Most commonly, the mosquitoes involved are *Aedes aegypti* and *Aedes albopictus*, two species which can also transmit other mosquito-borne viruses, including zika and chikungunya. Other infection routes are reported from mother to child as well as blood transmission (figure 11).

	Dengue	Zika	Chikungunya
Mosquitoes	*Aedes aegypti* *Aedes albopictus*	*Aedes aegypti* *Aedes albopictus*	*Aedes aegypti* *Aedes albopictus*
From mother to child	Evidence of transmission from an infected mother to her fetus	Rarely around time of birth, but it is possible that the virus could be passed to her fetus during pregnancy	Rarely from mother to newborn around the time of birth
Breastfeeding	No evidence	No evidence	No evidence
Blood	Rare cases known of transmission via blood transfusions from infected donors	Spread of the virus through blood transfusion have been reported	No evidence, but in theory possible
Sexual	No evidence	Spread of the virus through sexual contact have been reported	No evidence

Fig 11: Comparisment of Dengue, Zika and Chikungunya transmission routes

The main transmission is through mosquitoes that generally acquire the virus while feeding on the blood of an infected person. After virus incubation for eight to ten days, an infected mosquito is capable, during probing and blood feeding, of transmitting the virus for the rest of its life. There is no way to tell if a mosquito is carrying the dengue virus. Infected female mosquitoes may also transmit the virus to their offspring by transovarial (via the eggs) transmission, but the role of this in sustaining transmission of the virus to humans has not yet been defined.

Infected humans are the main carriers and multipliers of the virus, and serving as a source of the virus for uninfected mosquitoes. The virus circulates in the blood of infected humans for two to seven days, at approximately the same time that they have a fever (see also clinical symptoms). Aedes mosquitoes may acquire the virus when they feed on an individual during this period. In parts of South East Asia and Africa, the transmission cycle may also involve jungle primates that act as a reservoir for the virus (fig 12).

Dengue is most widely transmitted by the mosquito named *Aedes aegypti*. The *Aedes albopictus* mosquito and other *Aedes* species also transmit disease in specific areas. *Aedes polynesiensis*, *Aedes scutellaris* and *Aedes pseudoscutallaris* in the Pacific Islands and New Guinea. *Aedes polynesiensis* in the Society Islands and *Aedes niveus* in the Philippines.

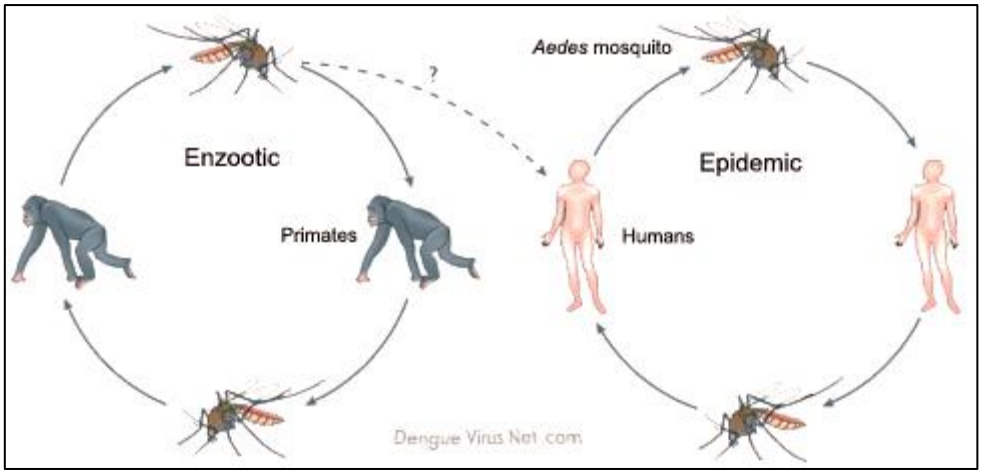


Fig 12. Transmission of dengue viruses.

The *Aedes* mosquito prefers to breed in water-filled receptacles, usually close to human habitation. They often rest in dark rooms (e.g. in bathrooms and under beds) and breed in small pools that collect in discarded human waste (see figure 3). Although they are most active during daylight hours, biting from dawn to dusk, mosquitoes will feed throughout the day indoors and during overcast weather. Dengue virus transmission follows two general patterns: epidemic dengue and hyperendemic dengue.

Epidemic dengue transmission occurs when dengue virus is introduced into a region as an isolated event that involves a single viral strain. If the number of vectors and susceptible pediatric and adult hosts is sufficient, explosive transmission can occur, with an infection incidence of 25-50%. Mosquito-control efforts, changes in weather, and herd immunity contribute to the control of these epidemics. This is the current pattern of transmission in parts of Africa and South America, areas of Asia where the virus has re-emerged, and small island nations. Travelers to these areas are at increased risk of acquiring dengue during these periods of epidemic transmission.

Hyperendemic dengue transmission is characterized by the continuous circulation of multiple viral serotypes in an area where a large pool of susceptible hosts and a competent vector (with or without seasonal variation) are constantly present. This is the predominant pattern of global transmission. In these populations, antibody prevalence increases with age and most adults are immune. Hyperendemic transmission appears to be a major risk for Dengue Haemorrhagic Fever (DHF). Travelers to these areas are more likely to be infected than are travelers to areas that experience only epidemic transmission.

The dengue fever is an acute febrile illness clinically characterized by haemorrhagic phenomenon and a tendency to develop a shock syndrome which may be fatal. The diagnostic symptoms are acute onset, high continuous fever lasting to 2-7 days, with various haemorrhagic manifestations like petechiae, purpura, achymosis epistaxis, gum-bleeding, hematemesis and/or melena, the enlargement of liver and shock manifested by rapid and weak pulse, narrow pulse pressure or hypotension, etc.

The patient is restless with cold clammy skin. Incubation period in man is 4-10 days. The mosquito becomes infected only during the first 3 days of patient's illness. Incubation period in mosquito varies from 8 to 11 days. Once infected, the mosquito remains so for its life and when the mosquito introduces saliva into the man's skin during feeding it transmits infection.

There is no transovarial transmission with respect to the mosquitoes. Although in India, it is becoming endemic in some parts, it still exists in epidemic form in certain parts of South-east Asia like

Thailand, Myanmar and Malaysia. The preventive measures include the vector control and the screening of all early cases so as to avoid the mosquitoes becoming infective.

Yellow fever

Yellow fever is a haemorrhagic condition that can lead to a high fever, bleeding into the skin, and cell death in the liver and kidneys. If enough liver cells die, liver damage occurs, leading to jaundice, a condition in which the skin takes on a yellowish colour.

It is an acute, systemic disease, meaning that it starts suddenly, and it affects the whole body. The Flavivirus causes yellow fever. The virus is transmitted by mosquitos, mainly the by *Aedes* species.

It is most likely to occur in tropical and subtropical parts of South America, parts of the Caribbean, and Africa. It rarely affects American travelers. The World Health Organization (WHO) estimate that in 2013, there were between 84,000 severe cases of yellow fever and between 29,000 and 60,000 fatalities. About 90 percent of all cases occur in Africa. An outbreak is currently underway in Brazil, and the Centers for Disease Control and Prevention (CDC) has in place a level 2 alert for travelers.

Transmission

Mosquitoes spread yellow fever. It can affect monkeys and humans.

If a mosquito bites a monkey that has the fever, they can pass it to humans. This can lead to outbreaks.

After having it once, a person is generally immune, meaning that they are unlikely to have it again.

Yellow fever can occur in settlements close to the jungle, where infected monkeys and mosquitoes live, and it can spread from there.

Symptoms

Most people with yellow fever do not develop symptoms, or the symptoms are very mild.

Yellow fever has an incubation period of between 3 and 6 days, so it takes from 3 to 6 days for signs and symptoms to appear after a person is infected. The disease cannot spread among humans. Only infection-carrying mosquitoes spread the disease to humans. The main symptoms of yellow fever are a high temperature, a slow pulse, albuminuria, jaundice, congestion of the face, and hemorrhage, or bleeding.

Signs and symptoms are categorized into two stages:

In the early, *acute stage*, the individual may experience:

- aching muscles, particularly the back and knees
- a high fever
- dizziness
- a headache
- loss of appetite
- nausea
- shivers, or chills
- vomiting

These symptoms usually disappear within 7 to 10 days.

- These symptoms usually improve after a few days, but around 15 percent of people enter a second stage, or toxic stage. The symptoms are more severe, and they may be life-threatening ecurring fever
- abdominal pain
- vomiting, sometimes with blood
- tiredness, sluggishness, lethargy
- jaundice, which gives the skin and whites of the eyes a yellow tinge
- kidney failure
- liver failure
- hemorrhage
- delirium, seizures, and sometimes coma
- arrhythmias, or irregular heartbeats
- bleeding from the nose, mouth, and eyes

Between 20% and 50 of people who develop toxic stage symptoms die within two weeks. Within 7 to 10 days, yellow fever is fatal in around half of all people who enter the toxic phase. Those who recover do not generally have any organ damage and are immune for life.

Morphological differences between *Anopheles, Culex* and *Aedes* mosquito

	Anopheles	***Culex***	***Aedes***
Adult			
1.	When it sits, the body makes an acute angle of 45^{o} with the substratum.	When it sits, the body placed more or less parallel to the substratum.	It sits more or less parallel to the substratum.
2.	Wings are provided with black and white bands.	Wing are devoid of any striation or bands.	Wings are large and thick and are provided with black and white bands.
3.	Scanty hairs are present on the antenna.	Dense hairs are present on the antenna.	Hairs are dense and coarse on the antenna.
4.	Both body and legs are narrow.	Both body and legs are comparatively broad.	Both body and legs are comparatively broad.
5.	Produce a peculiar sound during flight.	No sound is produced during flight.	Less sound is produced during flight.
Eggs			
6.	Eggs are small, boat like with air float.	Eggs are elongated and are devoid of air float.	Eggs are small and are devoid of air float.
7.	Eggs occur singly and no raft is formed.	Eggs occur in cluster forming a raft.	Eggs float separately and forming a raft.
8.	Number of eggs vary from 200-300.	Number of eggs vary from 200-800.	Number of eggs vary from 200-large number.

Larva			
9.	It floats horizontally placing its body parallel to the surface of water.	The larva floats obliquely with its head facing downwards.	Floats like *Culex* larva.
10.	Spiracles of 8th abdominal segment open on swellings placed side by side. Respiratory siphon is reduced in the larva.	Spiracles of 8th abdominal segment are placed on a pair of long tubular respiratory siphon.	There is tubular respiratory siphon on 9th abdominal segment.
Pupa			
11.	The pupa is green.	Colorless.	Colorless.
12.	Dorsal respiratory tube is small and flat.	Dorsal respiratory tube is narrow and elongated.	Same as *Culex* pupa
13.	Tail fin is large.	Tail fin is small.	Tail fin is medium.

Mosquito Control Measures

- **Personal protection:**

The eggs developing within the female mosquito need human blood for nourishment and so the female mosquito bites humans. By personal protection against mosquito bites, this blood meal can be denied, leading to reduction in mosquito eggs and hence mosquito population. Personal protection by covering the body with clothes and use of mosquito nets and repellents will further help in preventing mosquito bites.

Closure of windows and doors to prevent entry of mosquitoes inside house;

Protection of humans against mosquito bite by using bed nets (insecticide treated) and mosquito repellent.

- **Source reduction:**

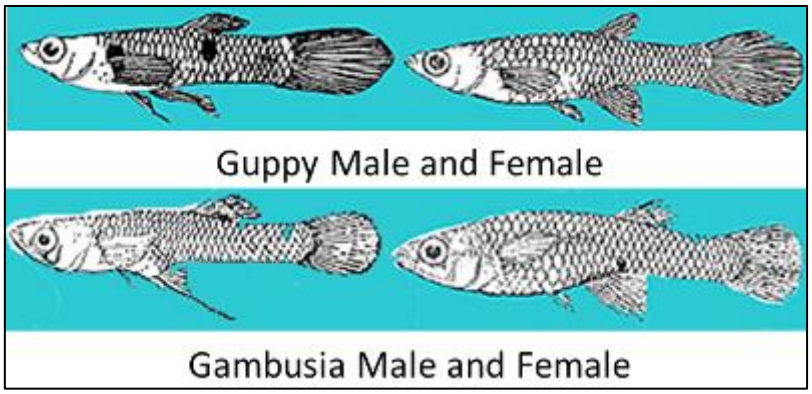
High humidity and ambient temperature between 20-30ºC provide ideal conditions for breeding of Anopheline mosquitoes. Common sites of breeding for *Anopheles* mosquitoes include rainwater pools and puddles, borrow pits, river bed pools, irrigation channels, seepages, rice fields, wells, pond margins, sluggish streams with sandy margins, hoof prints, tyre tracks etc. Water stagnation due to construction of dams, reforestation, shrimp farming, fish ponds etc., and have also been identified as possible sites of *Anopheles* breeding. *An. stephensi* is a well-adapted urban vector, being a container breeder, making use of man-made sites such as building-construction sites, wells, garden ponds, cisterns, overhead tanks, ground level cement tanks, water coolers, tyres, barrels and tins, intra-domestic containers etc. Prevention of water logging, destroying unwanted water collections and keeping the water containers closed, sources of egg laying (Source Reduction) can be denied and breeding of mosquitoes can be prevented.

- **Killing of the developing larvae and pupae:**

The best method of mosquito control is preventing the development of the eggs into adult mosquitoes, by reducing the sources of breeding. These anti larval measures are not only simple and cost effective, but also environment friendly.

Different types of chemical (insecticides) or biological (Guppy, *Poecilia reticulate* or Gambusia, *Gambusia affinis* fish or bacteria or fungii*)* larvicides (Themiphos and Fenthion are the two commonly used larvicidal agents. Oils may be applied to the water surface, suffocating the larvae and pupae.) can be used on such breeding grounds to kill the developing larvae and pupae.

- Bacteria such as *Bacillus sphaericus* and *Bacillus thuringiensis var israelensis* are also effective larvicides. However, they need to be re-introduced every 15 days and their culture may need expertise.
- Mermitid Nematod (*Romanomermis culicivorax*), Notonectid (Bug), Ambylospora (Protozoa), Coelomomyces (Fungus), Nuclear Polyhedrosis (Virus), and Cyclopoid copepods (Crustacean) are the other biological larvicides found to be effective.



- **Killing of the adult:**

Sprays are used to instantly kill the adults and residual sprays, on their resting places such as walls, are used for residual mosquitocidal effect.

a. Preventing egg laying: The easiest, cheapest and most environment-friendly method to control malaria is by preventing the mosquito from laying eggs. This is done by avoiding or eliminating the clean water collections.

To add to the problem, construction workers tend to harbour the malarial parasite, due to frequent infections owing to their poor standards of living. Thus, construction sites not only provide for mosquito breeding but also supply the parasites. This is the reason why malaria tends to be more common in cities where construction activities are in full swing.

Method # 1. Personal Protection:

(a) In mosquito-infected area protective clothing may be used, such as will cover the exposed parts of the body, especially after sunset,

(b) Mosquito repellents are also useful, like mosquito cream, citronella, odomos and Indalone which keep mosquitoes away. Repellent No. 448 of the American navy is very effective for long periods,

(c) While sleeping fine mesh mosquito nets prevent them from biting and bed rooms or houses could be screened to prevent entry of mosquitoes,

(d) Painting walls with creosote repels mosquitoes.

Method # 2. Destruction of Adults:

(a) Killing of mosquitoes can be done be spraying liquid insecticides like flit or D.D.T., the latter not only kills mosquitoes but also makes them leave a house,

(b) Fumigation of dwellings with sulphur dioxide is also useful,

c) A mixture of water and 10% D.D.T. in oil sprayed from the air is very effective in killing large number of mosquitoes in towns, ponds, marshes and forests.

Method # 3. Destruction of Larvae:

It is easier and more effective to kill mosquitoes in their larval forms than as adults, and several methods are used with success,

(a) Oiling:

The breeding places of mosquitoes are sprayed with petroleum oils, the oil film formed on the surface of water does not asphyxiate the larvae, as is commonly believed, but is toxic to them, the oiling must be repeated to kill those larvae and pupae which will hatch later.

(b) Panama Larvicide:

Panama larvicide is a mixture of caustic soda, resin, and phenol in water, it has been used most effectively in the Panama Canal region. The Panama larvicide mixes well with water and kills both the larvae and the algae on which they feed. One part of Panama larvicide is sufficient for 10,000 parts of water.

(c) Paris Green:

Paris green is a powder of arsenic mixed with fine dust, one part of powder with 100 parts of dust. This can be thrown in the wind and it will cover the surface of a pond; it is insoluble in water and remains floating and is eaten by surface feeding larvae of Anopheles', it will kill the larvae but not pupae. It is effective only against those larvae which feed on surface.

(d) Natural Enemies:

Fishes, minnows and *Gambusia* live on larvae and pupae of mosquitoes, and their introduction in a breeding place is helpful, but for this the brush and floating vegetation must be cleared so that the fish can reach the larvae.

(e) Chemical Larvicide:

One part of D.D.T. emulsion in thirty million parts of water is used most extensively as a spray to kill larvae, but it takes 50 hours. Planes can be used for this purpose on large areas.

Method # 4. Elimination of Breeding Places:

For those mosquitoes which breed in rain-filled containers and cisterns, like Aedes, emptying of water is effective. For large ponds and swamps digging a sloping ditch removes large volumes of water. Small ponds can be filled up with mud. In India cycles of 5 wet days followed by 2 to 4 dry days were found to be highly effective in controlling Anopheles in fields.

Method # 5. Preventive Medicine:

Daily doses of quinine are effective against mosquito bites, but a successful vaccine has yet to be found for yellow fever.

Probable questions:

1. What is the primary vector of malaria?
2. Describe the life cycle of *Anopheles* sp. With suitable diagram.
3. Write down the description of adult *Anopheles* mosquito.
4. Which type of malaria is most deadly type? Write the name of causative agent of this type of malaria.
5. Write the name of four types of malaria with its respective causative agents.
6. What is sporozoite?
7. What is cryptomerozoite?
8. What do you mean by scizont?
9. Describe the mode of malaria transmission.
10. Name the *culex* sp found in temperate region and tropical region.
11. Describe the morphology of adult *Culex* sp.
12. Describe the life cycle of *Wuchereria bancrofti* with diagram.
13. Discuss briefly the mode of transmission of filariasis.
14. What are the symptoms and treatment of filarial disease?
15. Describe the mode of transmission of those diseases which are transmitted by *Aedes* mosquito.
16. Describe the mode of transmission of Dengue virus.
17. Describe transovarial transmission with example.
18. Describe the mosquito control measures.

Suggested readings:

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Unit VIII

Life cycle, mode of transmission, control and importance of Ticks and mites

Objective: In this unit you will know about life cycle, mode of transmission, control and importance of Ticks and mites

Introduction

Ticks are ectoparasites, living by hematophagy on the blood of mammals, birds, and sometimes reptiles and amphibians to complete their complex life cycles. Ticks are small arachnids, part of the order Parasitiformes. Along with mites, they constitute the subclass Acaria. The fossil record suggests ticks have been around at least 90 million years. There are over 800 species of ticks throughout the world, but only two families of ticks, *Ixodidae* (hard ticks) and *Argasidae* (soft ticks), are known to transmit diseases or illness to humans. Hard ticks have a scutum, or hard plate, on their back while soft ticks do not. Tick-borne diseases occur worldwide.

Systematic position:

Kingdom-Animalia

Phylum-Arthropoda

Class-Arachnida

Subclass-Acaria (Acari, Acarina, Acarida)

Order-Anactinotrichidea (= Parasitoformes)

Suborder-Ixodida (= Metastigmata)

Family-Ixodidae

Families

- Ixodidae – hard ticks
- Argasidae – soft ticks
- Nuttalliellidae

Tick facts

- Ticks are scientifically classified as Arachnida (a classification that includes spiders). The fossil record suggests ticks have been around at least 90 million years.
- Most tick bites do not transmit harmful microbes.
- There are a variety of tick-borne diseases.
- There is a wide range of symptoms that usually develop days to weeks after the tick bite. The symptoms depend on the particular microbe that is transmitted.
- For all tick bites, local cleansing and antibiotic cream may be applied.
- There are safe and effective methods for the removal of all types of ticks.

Habit and Habitat of Ticks:

Ticks' importance as agents or vectors has long been recognized. In general, ticks are parasites of animals. Most species of vertebrates higher than fishes are subjected to attack by ticks, particularly mammals whose warm blood is highly attractive to ticks. They parasitize man and other domesticated mammals fortuitously.

The high potency of ticks in the spread of disease of man and animals are due to some factors like:

1. They are persistent blood suckers:
They attach firmly while feeding and cannot be dislodged easily. Most species have a wide range of host, thus ensuring a relatively certain source of blood.

2. Longevity:
Most species have a long life span, may be 5 years or more. It is helpful for them to carry the infection for several years.

3. High reproductive potential:
The reproductive potential is quite high. Some species may deposit as many as 18,000 eggs. Some have the power to regenerate lost parts.

General Morphology of Ticks:

Ticks are characterized by a leathery integument and larger size in comparison to mites. Body is segmented but without readily visible segments (Fig 1).

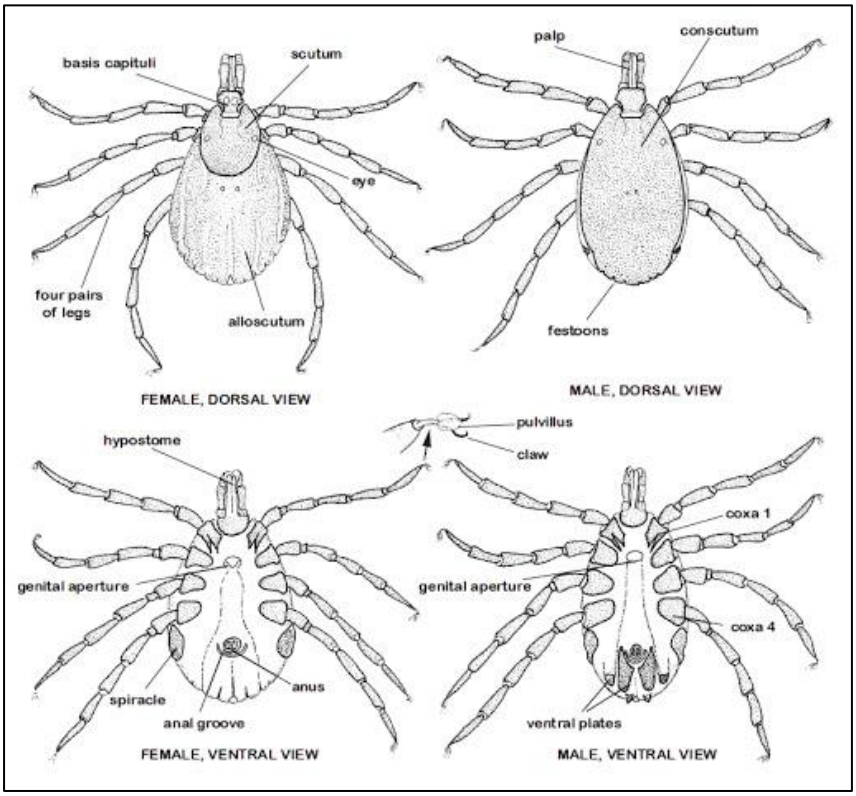


Fig 1: General morphology of Tick

The body is divided into two regions:

- The capitulum (also referred to as gnathosoma)
- The body proper

Capitulum is not the true head though it is commonly referred to as such. It projects anteroventrally and bears the mouth parts and a basal chitinous segment known as basis capitulum. The ring-like basis capitulum connects the capitulum to the body proper.

The mouth parts include 3 types of structures:

- Ventral to the mouth is toothed, elongated hypo-stoma, its free end projects anteriorly (Fig 2).
- On the dorsal surface of hypo-stoma is located a pair of chelicerae, on each side of the mouth. The free terminal of each chelicera is forked (chelate), giving rise to a dorsal, fixed toothed digitus externus and lateral movable digitus internus.
- The chelicerae function as piercing, tearing and anchoring structure by means of which the host's integument is opened and the entire capitulum or at least the toothed hypostome is inserted into the host.
- A pair of palpi or pedipalpi arises from the anteroventral margin of the basis capitulum. These structures act as counter-anchors while the tick is attached to the host. The mouth-parts show characteristic differences among different species and also between male and female of the same species

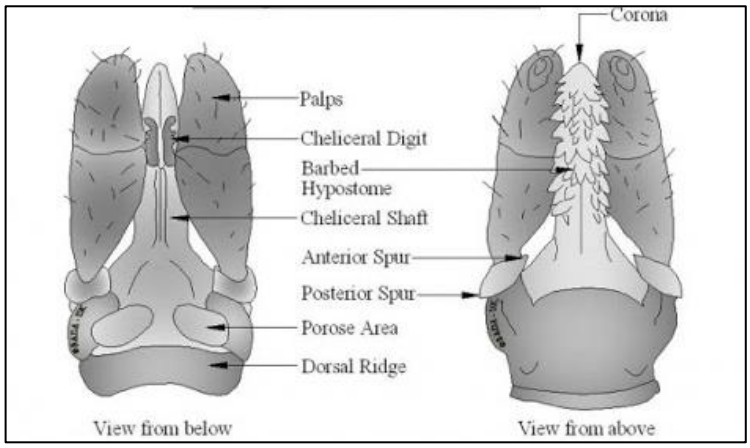


Fig 2: Mouthparts of tick

The *body proper* also shows differences among members of different families.

- It bears four pairs of legs; each is subdivided into six segments known as coxa, trochanter, femur, genu, tibia and tarsus. In some species, some of these units are fused. The legs characteristically terminate in a pair of claws on the tarsi.
- The genital orifice is located on the mid-ventro line between the first and second pairs of legs. The anus is also ventrally located, equidistant from the level of fourth pair of legs and the posterior margin of the body.
- On the basis of the difference present in body proper, two families are recognised in the order Ixodida; family Ixodidae and family Argasidae. Members of Ixodidae are commonly called **hard ticks** while those of argasidae are known as **soft ticks**. The morphological characteristics of these two families are discussed in Table 1.

Table 1: Differences between hard ticks and soft ticks

Characteristics	Argasidae	Ixodidae
MORPHOLOGY		
I. Capitulum		
Location	Sub terminal and so not visible on the dorsal aspect of adults Terminal and visible from dorsal aspect in larval stages	Terminal, visible from the dorsal aspect in all stages
Basis capitulum	Porose areas are absent	In female ticks the porose areas are seen on the dorsal aspect of basis capitulum
Pedipalps	Leg like, all 4 articles sub equal and flexible	Article IV recessed in a cavity in article III and the 1st article of the pedipalp is rigid
Chelicerae	Cheliceral sheath is smooth and distended	Cheliceral sheath is covered with teeth/ spines
II. Body		
Appearance of the surface	Leathery, mammillated or wrinkled	Smooth or superficially striated
Scutum	Absent	Present, entire dorsal surface covered in male ticks, half the surface covered in nymphs and larvae while in females only 1/3rd of the dorsal surface is covered by scutum
Festoons	Absent	Present
Eyes(when present)	Ventro-lateral on the supra coxal fold, 2 pairs, between 1st and 2nd and 2nd and 3rd pair of legs	Dorso-lateral in position, one pair is seen between the 1st and 2nd pair of legs
Spiracles	Small, seen between the 3rd and 4th coxae on ventral aspect	Seen on large spiracular plates posterior to the 4th coxae
Plates/grooves	Absent	Present
III. Legs		
Coxae	Unarmed	Armed, with internal and external spurs
Pulvilli	Rudimentary or absent	Present
IV. Sexual dimorphism		
	Not marked	Marked
BIOLOGICAL CHARACTERS		
Feeding habits	Intermittent feeders, rapid feeding in case of nymphs and adults, could also be slow in larval stages, cuticle expands to accommodate the blood meal but does not grow. They feed for few hours on the host and hence there is no formation of attachment cement	Takes a single blood meal over a period of days. Slow feeder and hence attachment to the host becomes essential, they secrete attachment cement Cuticle grows to accommodate the large blood meal

Nymphal instars	Many	Only one
Mating	Off the host Single sex pheromone	On the host with the exception of *Ixodes* spp Multiple pheromone
Reproduction	200-300 eggs deposited by the female after each feeding Several batches of eggs are laid, so females feed and oviposit repeatedly Eggs emerge from opening on ventral side Eggs are waxed by the Gene's organ	Following engorgement the mated female deposits around 10,000-20,000 eggs per tick Only one batch of eggs and following oviposition the female tick shrivels and dies Eggs emerge from the dorsal aspect through a slit in between the scutum and basis capitulum Initially covered by the secretion of the accessory glands of the vagina followed by the Gene's organ
Host seeking behavior	Nidiculous, seen in protected areas such as the nest, cave, burrow etc of the host	Non nidiculous, seen in pastures and other areas where the host frequents, it ambushes the host
Seasonal activity	Active throughout the year	Active during the warmer months of the year
Habitats	Sheltered environments	Open environments
Life span	Long lived, infrequent blood meals, numerous nymphal stages and high resistance to starvation are the causes for the increase in life span	Not so long lived. Less resistant to starvation, only one nymphal stage and single blood meal

Life History of Ticks:

In the life cycle of all ticks, four basic stages could be recognized — egg, larva, nymph and adult. The life cycle of ticks requires 6 weeks to 3 years to complete in different species.

a. Eggs:

The gravid females drop onto the ground, where she deposits the eggs in the soil or humus. Hard ticks lay eggs in a few hundreds or even thousands, all at one time. The soft ticks lay eggs in batches of 20 to 100 over a long period. The eggs hatch in 1 to 3 weeks.

b. Larvae:

In the summer, tick eggs hatch into larvae. Once they reach this life stage, the thousands of tiny larvae that were once eggs are ready and waiting for their first host to pass by. A tick needs to feed on the blood of a host during each life stage in order to pass onto the next. Larvae are not infectious at their time of hatching. However at this stage, they seek smaller mammals as their first host, and one of the most common mammals they feed on is the white-footed mouse. After a blood meal, it drops off, and in course of time it moults to become a nymph. The duration of the larval stage may vary from 3 to 13 days. The larva of ticks possesses 3 pairs of legs.

c. Nymphs:
The nymph resembles the adult in having 4 pairs of legs, but it has no genital pore. The nymph are all blood-suckers, and they attach themselves to suitable hosts for a blood meal. Members of Ixodidae have one nymphal instar, but argasids have as many as five.

Ticks, at the nymph stage, become the biggest concern during the spring as they begin to search for their next host when the warmer weather breaks. During the months of May, June, and July, nymphs will crawl up tall blades of grass and brush to reach their host. Ticks don't jump or fly. A nymph will wait for a desirable host to pass by and they'll latch onto it when they make contact. This is called questing.

d. Adults:
Adult ticks may live for a year or more. Soft ticks live longer than hard ticks. At the time of copulation, that occur on the host, the male ticks produce a spermatophore, which is placed under the genital operculum of the female.

Females usually require blood meal for egg production, although exceptions are there. Most opportunist ticks feed on a variety of hosts, while few are host-specific. Hard ticks can withstand a period of star-vation as long as 16 years.

The tick is called a one-host tick when all nymphal moulting through all instars occur on the same host, e.g., *Boophilus* sp. When the nymph drops off, moults to adult and attaches to another host, the tick is said a two-host tick. Most ixodids are three-host ticks whereas argasids with their multiple nymphal stages are many-host ticks. Use of such a series of hosts actually increases the opportunities for transmission of pathogens.

Tick Borne diseases:

Major tick-borne diseases include:

- ***Bacteria***
  - **<u>Lyme disease or Borreliosis</u>**
    - **Organism**: *Borrelia burgdorferi*
    - **Vector**: deer tick (*Ixodes scapularis* (=*I. dammini*), *I. pacificus*, *I. ricinus* (Europe), *I. persulcatus* (Asia))
    - **Endemic to**: North America and Eurasia
    - **Symptoms**: Fever, arthritis, neuroborreliosis, cranial nerve palsy, carditis, fatigue, and influenza-like illness.
    - **Treatment**: Antibiotics (Doxycycline in non-pregnant adults, Amoxicillin in pregnant adults and children)
    - **Mode of transmission**: Ticks can acquire *B burgdorferi* from feeding on an infected animal host during any of the three life-cycle stages. Unless the tick has fed on an infected host before feeding on a person, infection cannot result from that tick bite. Even if a tick that has previously fed on an infected animal and then feeds on an infected animal, the animal may not acquire the infection. Mice do not appear to develop Lyme disease, but they do carry the bacteria. They may be considered infested rather than infected. Deer also are incompetent hosts for *Borrelia*. Ticks carry *B burgdorferi* organisms in their midgut. The bacteria are introduced into the skin by a bite from an infected tick, and disease is

transmitted to humans as the spirochete is translocated from the gut to the s alivary glands and then to the person at the site of the bite

- **Relapsing fever** (Tick-borne relapsing fever, different from Lyme disease due to different *Borrelia* species and ticks)
  - **Organisms**: *Borrelia* species Such as *Borrelia hermsii*, *Borrelia parkeri*, *Borrelia duttoni*, *Borrelia miyamotoi*
  - **Vector**: *Ornithodoros* species [**Reservoir** - *Ornithodoros* ticks are considered the best reservoir of TBRF *Borrelia* for several reasons: they have an extremely long life span without blood feeding; they have the capacity to harbour TBRF *Borrelia* for several years; they are able to transmit TBRF *Borrelia* from tick to tick through trans-stadial, trans-ovarial and venereal modes; and because of hyper parasitism. Numerous vertebrates have been reported to be naturally infected by TBRF *Borrelia*, but few studies have tested their role as reservoirs. Because of their endophilic characteristics, *Ornithodoros* ticks commonly parasitize and infect small mammals, birds, reptiles or bats living in their underground habitat.]
  - **Regions**: Primarily in Africa, Spain, Saudi Arabia, Asia in and certain areas of Canada and the western United States
  - **Symptoms**: Relapsing fever typically presents as recurring high fevers, flu like symptoms, headaches, and muscular pain, with less common symptoms including rigors, joint pain, altered mentation, cough, sore throat, painful urination, and rash.
  - **Treatment**: antibiotics are the treatment for relapsing fever, with doxycycline, tetracycline, or erythromycin being the treatment of choice.
  - **Mode of transmission**: Tickborne relapsing fever is strictly transmitted by *Ornithodoros* ticks that are haematophagous at all growing stages. *Ornithodoros* ticks attach to their hosts for less than one hour, except for some larvae that can stay 1–2 days. During feeding, some Ornithodoros ticks (e.g., *O. sonrai*) produce local analgesia so that the tick bites are not noticed. Vertebrates and humans become infected through contamination of the feeding site by salivary and/or coxal secretions during the blood meals. Non-treated humans are supposed to be asymptomatic carriers over several years with resurgence of the pathogen and infectiousness during relapses. The persistence of spirochaetes in other vertebrates is quite unknown. Ticks are infected by TBRF during a blood meal on a spirochaetaemic vertebrate (rodent). *Borrelia* spread in all tissues, including ovaries (responsible for trans-ovarial transmission), salivary glands and excretory organs. Ticks are able to maintain TBRF Borrelia during their whole life span (5–10 years).

- **Typhus**
  - **Organism**: *Rickettsia prowazekii* [*Rickettsia prowazekii* causes epidemic typhus. *Rickettsia typhi* and, occasionally, *R. felis* cause endemic typhus and are transmitted to humans by vectors such as lice (mainly epidemic) and fleas (mainly endemic)].
  - **Vector**: Lice, fleas
    Risk factors include visiting or living in areas where rats, mice, and other animals have high populations (for example, disaster areas, poverty-stricken areas, refugee camps, jails) where vectors such as fleas and lice can carry the bacteria from the animals to infect humans.

- **Symptoms**: Typhus is a vector-borne bacterial disease; there are two types termed endemic and epidemic.
  Endemic typhus symptoms can include rash that begins on the body trunk and spreads, high fever, nausea, malaise, diarrhoea, and vomiting.
  Epidemic typhus has similar but more severe symptoms, including bleeding into the skin, delirium, hypotension, and death.
- **Treatment**: Antibiotics e.g., azithromycin , doxycycline, tetracycline or chloramphenicol are used to treat endemic and epidemic typhus.

  Good hygiene and clean living conditions that reduce or eliminate exposure to rats, mice, and other animals and the vectors that they carry (lice, fleas) can prevent or reduce one's risk for both types of typhus.

  There is no commercially available vaccine against either endemic or epidemic typhus.

- **Rocky Mountain Spotted Fever**
  - **Organism**: *Rickettsia rickettsii*
  - **Vector**: wood tick (*Dermacentor variabilis*), *D. andersoni*
  - **Region (US)**: East, South West
  - **Vector**: *Amblyomma cajennense*
  - **Region (Brazil)**: São Paulo, Rio de Janeiro, Minas Gerais.
  - **Symptoms**:Fever, headache, altered mental status, myalgia, and rash.
  - **Treatment**: Antibiotic therapy, typically consisting of doxycycline or tetracycline.
  - **Mode of transmission**: Ticks are the natural hosts of *R. rickettsii*, serving both as reservoirs and as vectors. The two major vectors of *R. rickettsii* in the United States are the American Dog tick (*Dermacentor variabilis*) and the Rocky Mountain Wood tick (*Dermacentor andersoni*). Ticks may obtain the pathogen by feeding on small mammals such as chipmunks and squirrels functioning as reservoirs for *R. rickettsii*. During feeding of larval and nymphal stages on these animals, ticks are most often infected with *R. rickettsii*. Once infected, ticks may spread the infectious agent trans-stadially among their own population, or trans-ovarially from the female tick to its offspring. The latter seems to be the primary way by which *R. rickettsii* propagates in nature. Dogs and humans may also function as reservoirs, but probably constitute incidental hosts and display clinical signs of disease. Ticks transmit the *rickettsiae* to all vertebrates primarily through their saliva during feeding. It usually takes several (5 to 20) hours of attachment and feeding before the *rickettsiae* are transmitted to the host. Less commonly, infections may occur following exposure to crushed tick tissues, fluids, or tick faeces.

- **Helvetica Spotted fever**
  - **Organism**: *Rickettsia helvetica*
  - **Region** (*R. Helvetica*): Confirmed common in ticks in Sweden, Switzerland, France and in Laos
  - **Vector**: *Ixodes ricinus* is the main European vector
  - **Symptoms**: Most often small red spots, other symptoms are fever, muscle pain, headache and respiratory problems
  - **Treatment**: Broad band Antibiotic therapy are needed, it is likely that phenoxymethyl penicillin is sufficient.

- **Ehrlichiosis anaplasmosis** (formerly Human Granulocytic Ehrlichiosis or HGE)
  - **Organism**: *Ehrlichia chaffeensis*, *E. equi* (renamed to *Anaplasma phagocytophilum*)
  - **Vector**: lone star tick (*Amblyomma americanum*), *I. scapularis*
  - **Region (US)**: South-Atlantic South-Central

- **Tularaemia**
  - **Organism**: *Francisella tularensis*, *A. americanum*
  - **Vector**: *D. andersoni*, *D. variabilis*
  - **Region (US)**: Southeast, South-Central, West, Widespread
  - **Symptoms**: Tularaemia causes fever, fatigue, aches and headache. Swollen lymph nodes are common. A sore may form at the site of inoculation. The organism may spread widely, causing major organs to fail. Pneumonia is common after inhalation but may also occur when the organism spreads throughout the body. If untreated, tularaemia is often fatal. With treatment, death is rare.
  - **Treatment**: Tularaemia is treated with intramuscular streptomycin or intravenous gentamicin. Oral medications are less reliable and are not currently recommended for significant disease.
  - **Mode of transmission**: Tularaemia affects animals and humans. Humans acquire tularaemia when they come into contact with infected animals or are bitten by insects that feed on infected animals. This disease may be spread through inhalation of dried animal matter, eating undercooked game, skinning or dressing killed animals, or drinking water contaminated with animal carcasses. It is not transferred from person to person.

➢ **Viruses**

- **Tick-borne meningoencephalitis**
  - **Organism**: TBEV aka FSME virus, a flavivirus from family Flaviviridae
  - **Vector**: deer tick (*Ixodes scapularis*), *Ixodes ricinus* (Europe), *Ixodes persulcatus* (Russia + Asia))
  - **Endemic to**: Europe and Northern Asia

- **Colorado tick fever**
  - **Organism**: Colorado Tick Fever virus (CTF), a coltivirus from Reoviridae
  - **Vector**: *Dermacentor andersoni*
  - **Region**: US (West)

- **Crimean-Congo hemorrhagic fever**
  - **Organism**: CCHF virus, a nairovirus, from Bunyaviridae
  - **Vector**: *Hyalomma marginatum*, *Rhipicephalus* bursa
  - **Region**: Southern part of Asia, Northern Africa, Southern Europe

- **Severe Febrile Illness**
  - **Organism**: Heartland virus, a phlebovirus, from Bunyaviridae

- **Vector***:* Lone Star Tick *(Amblyomma americanum)*
- **Region***:* Missouri and Tennessee, United States

➢ Protozoa

- **<u>Babesiosis</u>**
  - **Organism**: *Babesia microti, B. equi*
  - **Vector**: *Rhipicephalus sanguineus , Dermacentor* ticks, *Ixodes scapularis*
  - **Region (US)**: Northeast West Coast
  - **Mode of transmission**: *Rhipicephalus sanguineus* is the primary vector for *Babesia* in warmer regions worldwide, like in Southern Europe, Southern USA, Australia and Latin America. In Western and Central Europe, the main vectors for *Babesia* are *Dermacentor* ticks, esp. *Dermacentor reticulatus*. Transmission within the tick is both trans-stadial (infection at any stage for *Rhipicephalus* and the next stage being infectious) and trans-ovarial (females of *Rhipicephalus* and *Dermacentor* may transfer infection to the next generation through eggs). As a consequence, nymphs and adults of *Rhipicephalus* can be infectious when larvae or nymphs have fed on an infected dog (trans-stadial), whereas adults of *Dermacentor* will only be infectious from the previous infected tick generation (trans-ovarial), because larvae and nymphs of *Dermacentor* do not feed on dogs.

    Babesia microti parasites live in the gut of the black-legged or deer tick (*Ixodes scapularis*). The tick attaches to the body of white-footed mice and other small mammals, transmitting the parasite to the rodents' blood. After the tick has eaten its meal of the animal's blood, it falls off and waits to be picked up by another animal. The white-tailed deer is a common carrier of the deer tick. The deer itself isn't infected. After falling off the deer, the tick will typically rest on a blade of grass, a low branch, or leaf litter. If you brush up against it, it can attach to your shoe, sock, or other piece of clothing. The tick then climbs upward, seeking a patch of open skin. Human probably won't feel the tick bite, and you may not even see it. That's because most human infections are spread during spring and summer by ticks in the nymph stage. During this stage, the ticks are about the size and colour of a poppy seed.

  - **Symptoms:** High fever, chills, muscle or joint aches, and fatigue. Less common symptoms include - severe headache, abdominal pain, nausea, skin bruising, yellowing of your skin and eyes, mood changes

- **<u>Cytauxzoonosis</u>**
  - **Organism**: *C. felis*
  - **Vector**: *D. variabilis* (American Dog Tick)
  - **Region (US)**: South, Southeast

MITES

Mites are among the smallest arthropods with most barely visible without magnification. Mites are closely related to ticks, but they are tissue-juice feeders, not blood-feeders, and do not transmit as broad a variety of infectious microbial diseases. In fact, the only infectious diseases transmitted by mites are rickettsial pox and scrub typhus. The most common ectoparasitic dermatoses caused by mites are chiggers and scabies.

General Morphology of mites:

External

Mites are tiny members of the class Arachnida; most are in the size range 0.25 to 0.75 mm (0.01 to 0.03 in) but some are larger and some are no bigger than 0.1 mm (0.004 in) as adults. The body plan is similar to that of ticks in having two regions, a cephalothorax (with no separate head) or prosoma, and an opisthosoma or abdomen (Fig 3). Segmentation has almost entirely been lost and the prosoma and opisthosoma are fused, only the positioning of the limbs indicating the location of the segments.

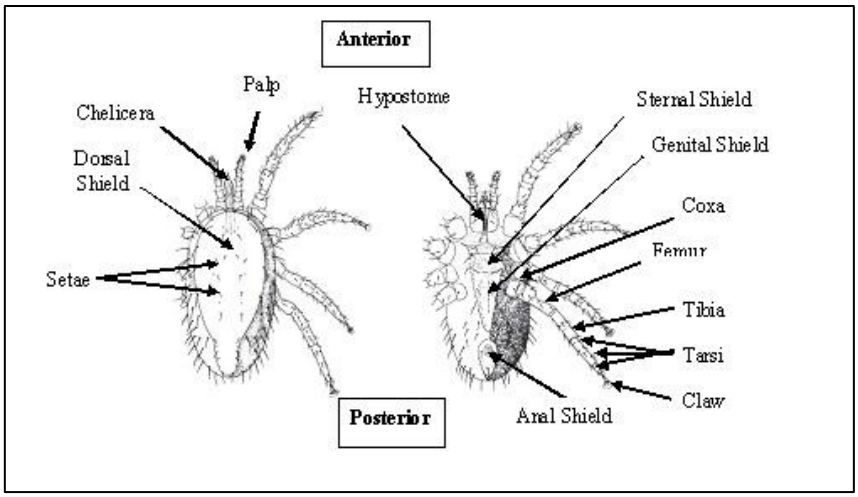


Fig 3: General structure of mite

The body is covered with tactile hairs or scales. There is no true head, but the mouth parts are borne on an anterior part, called a gnathosoma or capitulum. This is not a head and does not contain the eyes or the brain, but is a retractable feeding apparatus consisting of the chelicerae, the pedipalps and the oral cavity. It is covered above by an extension of the body carapace and is connected to the body by a flexible section of cuticle. There are two pairs of mouth parts, the chelicerae and the pedipalps or palpi. The mouthparts differ between taxa depending on diet; in some species the appendages resemble legs while in others they are modified into chelicerae-like structures. The oral cavity connects posteriorly to the mouth and pharynx. Eyes may be present or absent. Most mites have four pairs of legs, each with six segments, which may be modified for swimming or other purposes. The dorsal surface of the body is clad in hardened tergites and the ventral surface by hardened sclerites; sometimes these form transverse ridges. The gonopore (genital opening) is located on the ventral surface between the fourth pair of legs. Some species have one to five median or lateral eyes but many species are blind, and slit and pit sense organs are common. Both body and limbs bear setae (bristles) which may be simple, flattened, club-shaped or sensory. Mites are usually some shade of brown, but some species are red, orange, black or green, or some combination of these colours.

Internal

Mites have a typical arachnid digestive system, although some species lack an anus: they do not defecate during their short lives. The circulatory system consists of a network of sinuses and lacks a heart, movement of fluid being driven by the contraction of body muscles. Gas exchange is carried out across the body surface, but many species additionally have between one and four pairs of tracheae, the spiracles being located in the front half of the body. The excretory system includes a nephridium and one or two pairs of Malpighian tubules.

Life History of Mites:

Many mites are free living, some are predaceous and many are parasitic on other animals during all or part of their life cycle. Some of these rank among the most important disease vectors and some act as intermediate hosts of Protozoans or Helminths.

There are usually four stages in the development (i.e. life history) of mites—egg, larva, nymph and adult. However, there are usually a single larval stage and two nymphal stages (i.e., the protonymph and deutonymph) in the life cycle of a mite. The number of nymphal generation may be less or more in some species of mites.The sexes are separate in mites; males have a pair of testes in the mid-region of the body, each connected to the gonopore by a vas deferens, and in some species there is a chitinous penis; females have a single ovary connected to the gonopore by an oviduct, as well as a seminal receptacle for the storage of sperm. In most mites, sperm is transferred to the female indirectly; the male either deposits a spermatophore on a surface from which it is picked up the female, or he uses his chelicerae or third pair of legs to insert it into the female's gonopore. In some of the Acariformes, insemination is direct using the male's penis.

The eggs are laid in the substrate, or wherever the mite happens to live. They take from two to six weeks to hatch, and the first stage larvae have six legs. After three moults, the larvae become nymphs, with eight legs, and after a further three moults, they become adults. Longevity varies between species, but the lifespan of mites is short as compared to many other arachnids.

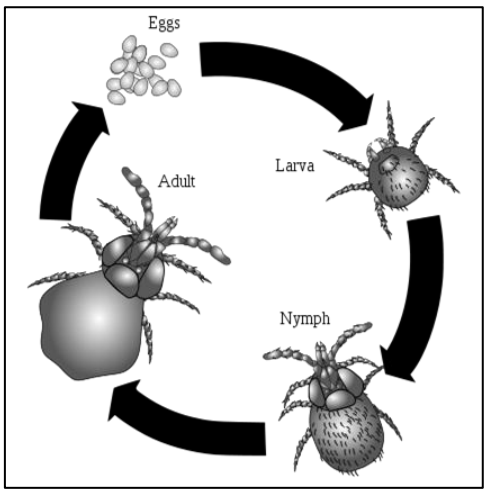


Fig 4: Life cycle of mite

General Idea about Mites as Vectors:

There are some species of mites which are important from the stand point of veterinary science as they cause transmission of several diseases. There are also few species of mites which help in the transmission of human diseases including allergy.

The important vector species of mites will be discussed:

1. Redbugs:

There is probably no creature on earth that can cause more torment for its size than a red bug. These mites are also suspected on epidemiological grounds of transmitting epidemic hemorrhagic fever.

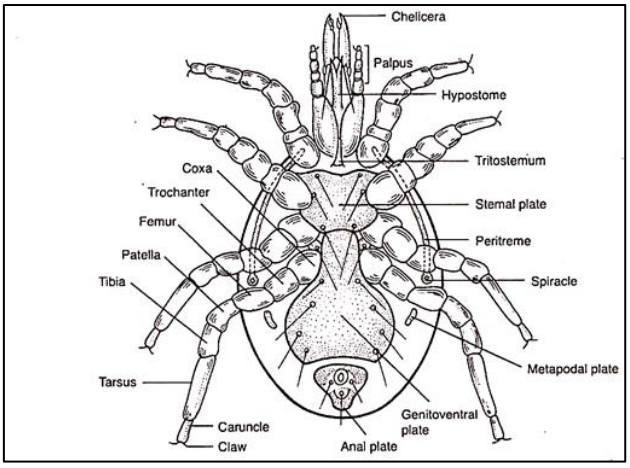


Fig 5: Generalized Mesostigmatide Mite

The red bugs are the six-legged larvae of mites of the family Trombiculidae under order Prostimata, comprise a group of mites which are parasitic to human beings only at their larval stage. The nymphs and adults are free living, feeding on insect eggs or minute insect larvae. In Siberia, Korea, Manchuria scrub typhus is a viral disease which causes fever, kidney damage etc. and is fatal in 5% of cases.The important chigger mite, *Trombicula akamushi* is responsible for causing scrub typhus to human beings but *T. pelkini*, *T. goldii*, *T. wichtnanni* etc. create human dermatitis.

The larval mite acts as the vector in carrying the pathogen, *Rickettsia tsutsugamushi* causing scrub typhus. They transmit the pathogen from small mammals to human beings (hosts). The larval mites receive the pathogens by means of trans-ovarian transmission from the mother.

Now the larvae penetrate the epidermis by means of mouth parts and introduce the salivary secretion containing the pathogen into the host. The Escher is the primary lesion which appears just at the point of red-bug bite.

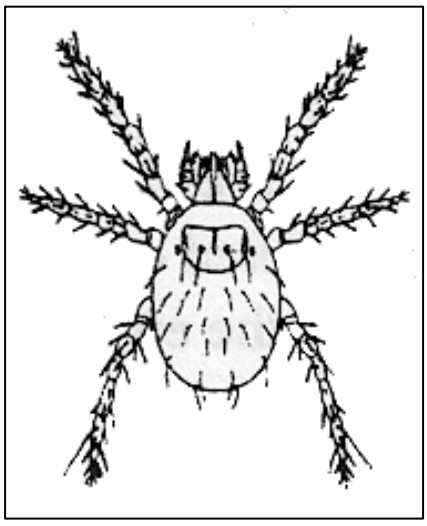
It is characterized by headache, apathy, fever, lymph adenitis, general malaise, enlarged spleen, deafness, nervous turbances etc. The Escher enlarges and becomes necrotic in the center and red rashes appear on the trunk that may spread to extremities.

2. Chiggers

Chiggers are the larvae of a family of mites that are sometimes called red bugs. The adults are large, red mites often seen running over pavement and lawns. Chiggers are extremely small (0.5 mm) and are difficult to see without magnification. The six-legged larvae are hairy and yellow-orange or light red. They are usually encountered outdoors in low, damp places where vegetation is rank and grass and weeds are overgrown. Some species also infest drier areas, however, making it difficult to predict where an infestation will occur.

Chiggers overwinter as adults in the soil, becoming active in the spring. Eggs are laid on the soil. After hatching, the larvae crawl about until they locate and attach to a suitable host. The larvae do not burrow into the skin, but inject a salivary fluid which produces a hardened, raised area around them. Body fluids from the host are withdrawn through a feeding tube. Larvae feed for about 4 days and then drop off and molt to nonparasitic nymphs and adults. Chiggers feed on a variety of wild and domestic animals, as well as humans. The life cycle (from egg to egg) is completed in about 50 days.

Most people react to chigger bites by developing reddish welts within 24 hours. Intense itching accompanies the welts, which may persist for a week or longer if not treated. Bites commonly occur around the ankles, waistline, armpits, or other areas where clothing fits tightly against the skin. Besides causing intense itching, chigger bites that are scratched may result in infection and sometimes fever. Chiggers in North America are not known to transmit disease.



Persons walking in chigger-infested areas can be protected by treating clothing (cuffs, socks, waistline, and sleeves) or exposed skin with tick repellents. Some repellents should only be used on clothing; and it is important to follow label directions. People who suspect they may have been attacked by chiggers should take a soapy bath immediately and apply antiseptic to any welts. A local anesthetic will provide temporary relief from itching.

Regular mowing and removal of weeds and brush make areas less suitable for chiggers and their wild hosts. Mowing also enhances penetration and performance of miticides, should they be required. Chigger populations can be further reduced by treating infested areas with residual miticides. Applications should be thorough but restricted to areas frequented and suspected of being infested.

3. Itch and Eczema creating mites (*Sarcoptes scabiei*):

The minute rounded or oval, short legged, flattened mites of the family Sarcoptidae under the suborder Sarcoptiformes are the cause of scabies or “itch” in man. This mite creates eczema and unbearable itches leading to mange of man.

The cuticle of the mite is delicately sculptured, number of bristles is few, eyes and trachea are absent. Capitulum is well developed. The legs are short and stumpy and are provided with sucker like adhesive pads (Fig 6 and 7).

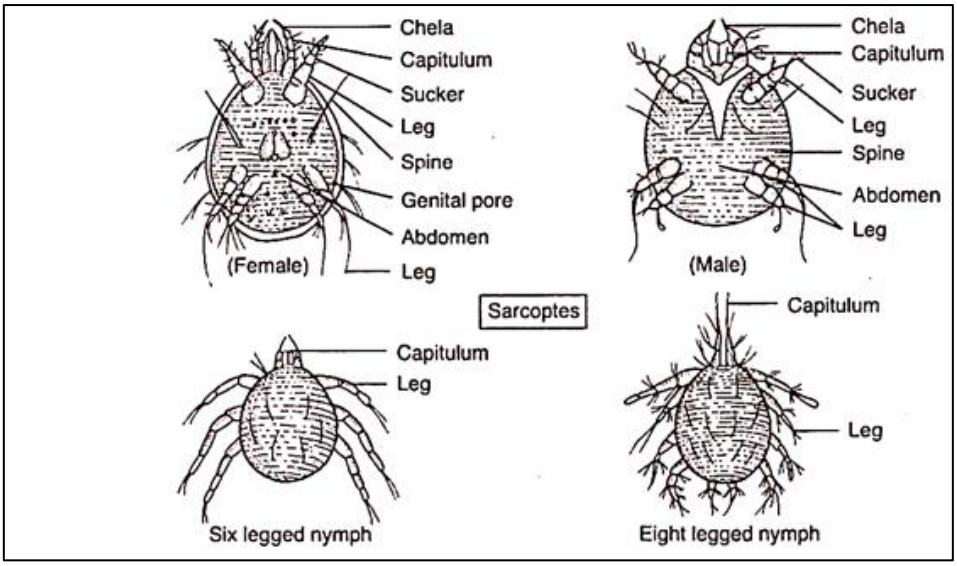


Fig 6: Structure of *Sarcoptes scabiei*

When the female mite, comes in contact with the hands or feet of human, it excavates thin tortuous tunnels in the epidermis. The tunnel mea-sures a few mm to over an inch in length and is usually gray from the eggs and excrement deposited by the female as she burrows. The daily excavations of a mite is about 2-3 mm.

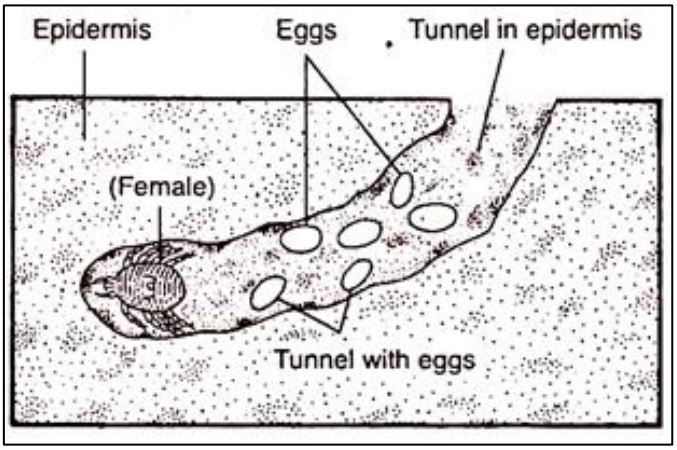


Fig 7: Tunnel of Itch Mite in Human Skin, showing female depositing egg

The life span of a mite is about 4 weeks and the young impregnated females make fresh excavations of their own and the process goes on. As a result the tissues below the epidermis are destroyed and finally itching begins. The itching is so severe that the patient cannot sleep at night and sometimes the infection becomes unbearable.

Repeated infection of itching turns into eczema. *Sarcoptes scabiei* generally invades the skin of the wrist though external genetalia, breasts, legs, thorax and other organs may be attacked by this mite.

4. The Tropical rat mite: The tropical rat mite, *Ornithonyssus bacoti* (formerly *Liponyssus* or *Bdellonyssus*) are important parasites of birds and rodents and are concerned in transmission of certain rickettsial and viral diseases. They have relatively narrow dorsal shields and che-licerae that end in pincers in both sexes.

They act as vectors of Q. fever (query fever) by transmitting the pathogen, *Coxiella bumetti*. The blood sucking protonymph is the infective stage of the disease. This tropical rat mite plays a minor role in transmission among reservoir hosts, and occasionally to human beings, of endemic typhus, rickettsial pox, Q. fever, plague, etc. and it also acts as the intermediate host of the filaria of cotton rats, *Liptomosoides*.

5. Human biting mites:

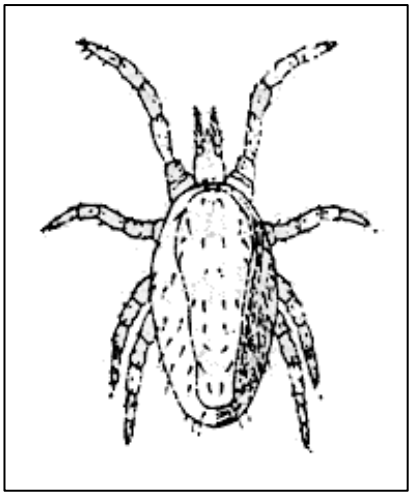
Several types of mites are associated with cases of skin dermatitis in humans. The tropical rat mite, *Ornithonyssus bacoti*, is one of the most common house invading species. The tropical fowl mite, *Ornithonyssus bursa*, and northern fowl mite, *Ornithonyssus sylviarum*, are also frequently encountered in homes. The latter two species are found principally on domestic or wild birds. The house mouse mite, *Liponyssoides sanguineus*, may also be found in structures with house mouse infestations. The tropical rat mite is a parasite on rats. Although none of these species are truly parasitic on humans, they bite people readily, often producing dermatitis and itching.

Rat and bird mite infestations occur in structures where rat or bird nests are located. Infestations are sometimes first noticed following extermination, or after the natural hosts have died or left the structure. Infestations may also occur where heavy mite infestations have developed around a rodent or bird nest. Occasionally rodent or bird mites may be found on rodents kept as pets.

Rat mites are small, approximately the size of the period at the end of this sentence. They move actively and can be picked up with a wet finger, brush or piece of sticky tape. Distinguishing between different species of *Ornithonyssus* mites to determine whether birds or rodents are the likely source is difficult and requires special expertise. The first course of action when faced with a suspected biting mite problem is to look for all potential bird or rodent sources and collect some of the mites, if possible.

6. Bird and Rodent mites

Parasitic mites that occasionally infest buildings are usually associated with wild or domestic birds or rodents. Bird and rodent mites normally live on the host or in their nests, but migrate to other areas of the structure when the animal dies or abandons the nest. Rodent mites often become a nuisance after an infestation of mice or rats has been eliminated. People usually become aware of the problem when they are attacked by mites searching for an alternate food source. Their bites cause moderate to intense itching and irritation. Rodent and bird mites are very tiny, but usually can be seen with the naked eye. They are about the size of the period at the end of this sentence.



The first step in controlling bird or rodent mites is to eliminate the host animals and remove their nesting sites. Often, the nests will be found in the attic, around the eaves and rafters, or in the gutters or chimney. Gloves should be used when handling dead animals. A respirator should also be worn when removing nest materials to avoid inhaling fungal spores and other potential disease-producing organisms associated with the droppings.

After nests are removed, the areas adjacent to the nest should be sprayed or dusted with a residual insecticide such as those products labeled for flea control. Space or ULV treatments with non-residual materials (e.g., synergized pyrethrins) can be used in conjunction with residual sprays. Space treatments are especially useful when the mite infestation has

dispersed widely from the nesting site. In this case, more extensive treatment with residual and non-residual insecticides may also be necessary in other areas of the structure where mites are observed. A vacuum cleaner or cloth moistened with alcohol can be used to eliminate mites crawling on open surfaces.

Mites are tiny arthropods, related to ticks. Several types of mites can be found in homes and of these a few may bite humans. Most mites are harmless predators of insects, or feeders on decaying plant material. Some pest mites feed on stored products like cheese and grain. Others are merely nuisance pests, accidentally entering homes from their normal outdoor habitat. Only a few mite species are parasitic on birds or mammals, but these can occasionally become biting pests in homes. Identifying the type of mite and/or likely host is the first step in solving an indoor mite infestation.

Disease transmitted by mites:

1. Scabies

Scabies is a skin infestation caused by a mite known as the *Sarcoptes scabiei*. These microscopic mites can live on your skin for months. They reproduce on the surface of your skin and then burrow into the upper layer of the skin but never below the stratum corneum and lay eggs. The burrows appear as tiny raised serpentine lines that are grayish or skin-colored and can be a centimeter or more in length. This causes an itchy, red rash to form on the skin. Other races of scabies mites may cause infestations in other mammals, such as domestic cats, dogs, pigs, and horses.

There are approximately 130 million Trusted Source cases of scabies in the world at any given time. While it's a highly contagious condition that can easily be passed from one person to another through direct skin contact, scabies isn't a sexually transmitted disease. The infestation of mites may also be transmitted through infested clothing or bedding. Intimate contact isn't necessary. Although scabies can be bothersome, they can usually be eliminated effectively.

Causal Agent:

Sarcoptes scabiei var. *hominis*, the human itch mite, is in the arthropod class Arachnida, subclass Acari, family Sarcoptidae.

It should be noted that races of mites found on other animals may cause a self-limited infestation in humans with temporary itching due to dermatitis; however they do not multiply on the human host.

Life Cycle:

Sarcoptes scabiei undergoes four stages in its life cycle: egg, larva, nymph and adult (Fig 8).

Eggs: Females deposit 2-3 eggs per day as they burrow under the skin. Eggs are oval and 0.10 to 0.15 mm in length and hatch in 3 to 4 days. After the eggs hatch, the larvae migrate to the skin surface and burrow into the intact stratum corneum to construct almost invisible, short burrows called molting pouches.

Larva: The larval stage, which emerges from the eggs, has only 3 pairs of legs and lasts about 3 to 4 days. After the larvae molt, the resulting nymphs have 4 pairs of legs. This form molts into slightly larger nymphs before molting into adults.

Nymph: Larvae and nymphs may often be found in molting pouches or in hair follicles and look similar to adults, only smaller.

Adult: Adults are round, sac-like eyeless mites. Females are 0.30 to 0.45 mm long and 0.25 to 0.35 mm wide, and males are slightly more than half that size.

Mating occurs after the active male penetrates the molting pouch of the adult female. Mating takes place only once and leaves the female fertile for the rest of her life. Impregnated females leave their molting pouches and wander on the surface of the skin until they find a suitable site for a permanent burrow. While on the skin's surface, mites hold onto the skin using sucker-like pulvilli attached to the two most anterior pairs of legs. When the impregnated female mite finds a suitable location, it begins to make its characteristic serpentine burrow, laying eggs in the process. After the impregnated female burrows into the skin, she remains there and continues to lengthen her burrow and lay eggs for the rest of her life (1-2 months). Under the most favorable of conditions, about 10% of her eggs eventually give rise to adult mites. Males are rarely seen; they make temporary shallow pits in the skin to feed until they locate a female's burrow and mate.

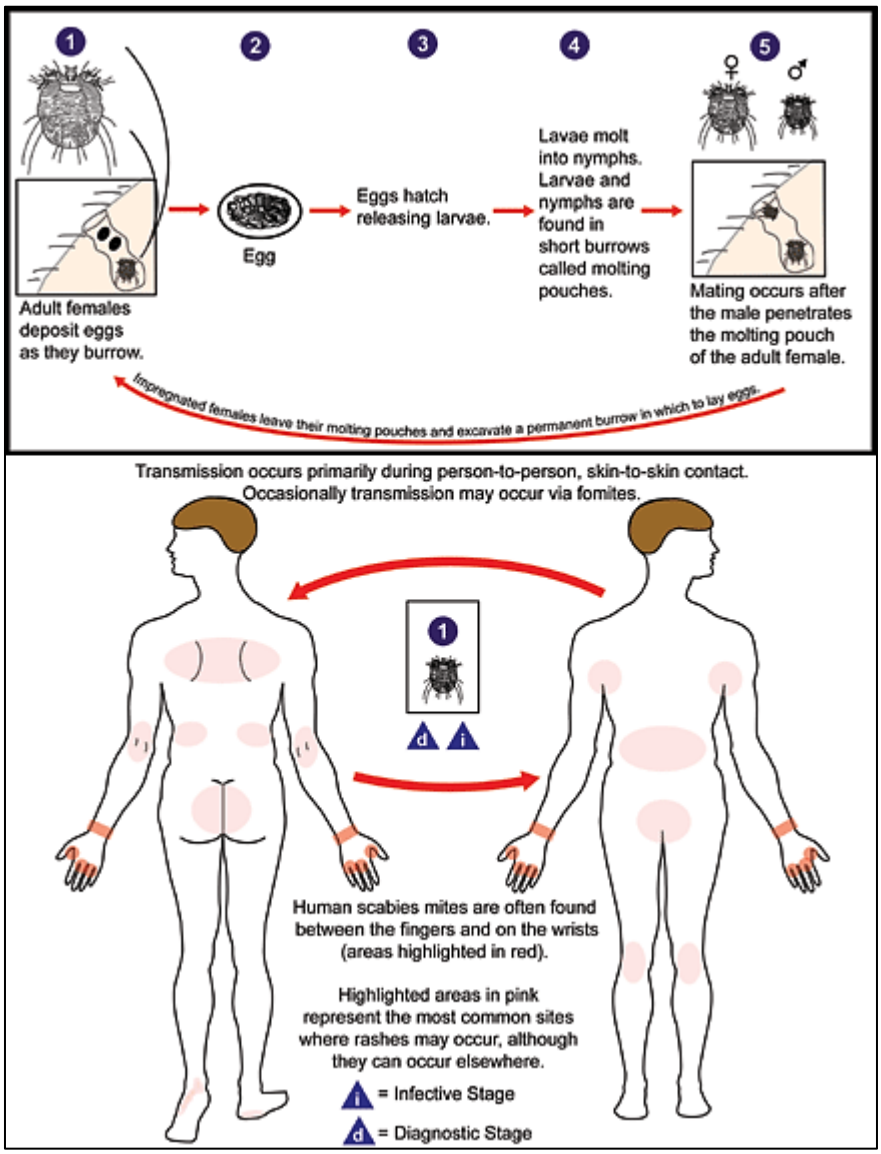


Fig 8: Life cycle of *Sarcoptes scabie*

Types of scabies

There's only one type of mite that causes a scabies infestation. This mite is called *Sarcoptes scabiei.* However, these mites can cause several types of infestations.

1. *Typical scabies*

This infestation is the most common. It causes an itchy rash on the hands, wrists, and other common spots. However, it doesn't infest the scalp or face.

2. *Nodular scabies*

This type of scabies may develop as itchy, raised bumps or lumps, especially in the genital areas, armpits, or groin.

3. Norwegian scabies

Some people with scabies may develop another form of scabies known as Norwegian scabies, or crusted scabies. This is a more severe and extremely contagious type of scabies. People with crusted scabies develop thick crusts of skin that contain thousands of mites and eggs.

Crusted scabies usually develops in people with weakened immune systems. This includes people with HIV or AIDS, people who use steroids or certain medications (such as some for rheumatoid arthritis), or people who are undergoing chemotherapy.

The scabies mites can overpower the immune system more easily and multiply at a quicker rate. Crusted scabies spreads in the same way as normal scabies.

Mode of transmission:

Transmission occurs primarily by the transfer of the impregnated females during person-to-person, skin-to-skin contact. Occasionally transmission may occur via fomites (e.g., bedding or clothing). Human scabies mites often are found between the fingers and on the wrists.

Common Symptoms

- ✓ The most common symptoms of scabies, itching and a skin rash, are caused by sensitization (a type of "allergic" reaction) to the proteins and faeces of the parasite. Severe itching (pruritus), especially at night, is the earliest and most common symptom of scabies. A pimple-like itchy (pruritic) "scabies rash" is also common.
- ✓ Itching and rash may affect much of the body or be limited to common sites such as: between the fingers, wrist, elbow, armpit, penis, nipple, waist, buttocks, and shoulder blades. The head, face, neck, palms, and soles often are involved in infants and very young children, but usually not adults and older children.
- ✓ Tiny burrows sometimes are seen on the skin; these are caused by the female scabies mite tunneling just beneath the surface of the skin. These burrows appear as tiny raised and crooked (serpiginous) grayish-white or skin-colored lines on the skin surface. Because mites are often few in number (only 10-15 mites per person), these burrows may be difficult to find.

Possible Complications

The intense itching of scabies leads to scratching that can lead to skin sores. The sores sometimes become infected with bacteria on the skin, such as Staphylococcus *aureus* or beta-hemolytic

streptococci. Sometimes the bacterial skin infection can lead an inflammation of the kidneys called post-streptococcal glomerulonephritis.

Treatment

Often consists of medications that kill scabies mites and their eggs. Since scabies is so contagious, doctors will usually recommend treatment for an entire group of people who are in frequent contact with a person who has scabies.

Recognizing scabies bites and the distinctive red rash can help you find treatment faster.

Application of ointments, creams, and lotions that can be applied directly to the skin. Oral medications are also available.

Your doctor will probably instruct you to apply the medicine at night when the mites are most active. You may need to treat all of your skin from the neck down. The medicine can be washed off the following morning.

Make sure you follow your doctor's instructions very carefully. You may need to repeat the topical treatment in seven days.

Some common medicines used to treat scabies include:

- 5 percent permethrin cream
- 25 percent benzyl benzoate lotion
- 10 percent sulfur ointment
- 10 percent crotamiton cream
- antihistamines, such as Benadryl (diphenhydramine) or pramoxine lotion to help control the itching
- An oral tablet called ivermectin (Stromectol) can be given to people who don't see an improvement in symptoms after initial treatment
- Sulfur is an ingredient used in several prescription scabies treatments. You can also purchase sulfur over the counter and use it as a soap, ointment, shampoo, or liquid to treat scabies.

Natural treatment of scabies

Common natural treatments for scabies include: Tea tree oil, Aloe vera, Capsaicin cream, Essential oils, Soaps

Prevention & Control

When a person is infested with scabies mites the first time, symptoms may not appear for up to two months after being infested. However, an infected person can transmit scabies, even if they do not have symptoms. Scabies usually is passed by direct, prolonged skin-to-skin contact with an infected person. However, a person with crusted (Norwegian) scabies can spread the infestation by brief skin-to-skin contact or by exposure to bedding, clothing, or even furniture that he/she has used.

Scabies is prevented by avoiding direct skin-to-skin contact with an infected person or with items such as clothing or bedding used by an infected person. Scabies treatment usually is recommended for members of the same household, particularly for those who have had prolonged skin-to-skin contact. All household members and other potentially exposed persons should be treated at the same time as the infested person to prevent possible re-exposure and reinfection. Bedding and clothing worn or used next to the skin anytime during the 3 days before treatment should be machine washed and dried using the hot water and hot dryer cycles or be dry-cleaned. Items that cannot be dry-cleaned or laundered can be disinfested by storing in a closed plastic bag for several days to a week. Scabies mites generally do not survive more than 2 to 3 days away from human skin. Children and adults usually can return to child care, school, or work the day after treatment.

Persons with crusted scabies and their close contacts, including household members, should be treated rapidly and aggressively to avoid outbreaks. Institutional outbreaks can be difficult to control and require a rapid, aggressive, and sustained response.

Rooms used by a patient with crusted scabies should be thoroughly cleaned and vacuumed after use. Environmental disinfestation using pesticide sprays or fogs generally is unnecessary and is discouraged.

Collecting mites

Most pest control companies will (rightly) not treat a home without proof of pest presence. It is therefore important to collect mites prior to treatment. Parasitic mites are often first noticed when biting. Mites can be collected from the skin with an artist's brush or tissue dipped in rubbing alcohol. Mites collected in this way should be placed in a small vial or other waterproof container with a small amount of rubbing alcohol. Mites can also be collected from the skin with a piece of tape (although this makes accurate identification of the mite unlikely). Sticky traps are also useful tools for sampling tiny arthropods around the home. Place several sticky traps in rooms where bites are occurring.

Control of mites

- ✓ The primary mite *host* must be eliminated before successful control of *rodent or bird mites* can be achieved. Clues to the type of host that has invaded the house can be deduced by the time of year that the mite infestation occurs. Rodent infestations are possible at any time of year, though they seem to occur most frequently in the fall and winter. Bird problems are most common during the spring and summer.
- ✓ To seal homes against rodents all vents and electric service entry points should be tightly closed with rodent-proof metal hardware cloth, metal flashing, or copper wool. Entry points around chimneys and between loose shingles should also be checked. Doors and windows should seal tightly. House mice will enter structures near the ground, especially under poorly-sealed doors. Rodent proofing must include the smallest entry holes. Mice can enter a home through a hole as small as a dime; rats can enter through a hole as small as a quarter.
- ✓ *Bird infestations* are often first indicated by the sound of chirping coming from a chimney or soffit area. The same rules and materials used for rodent-proofing are effective in keeping birds out of the home. Special screening may be needed on chimneys to deny bird's access to chimney areas. Birds nesting in chimneys may also indicate the need for chimney maintenance and cleaning.
- ✓ Chickens and other fowl kept in sheds or coops attached to a home can also be a source of mites indoors.
- ✓ Pesticides can help suppress mite populations in the home, but must be used in combination with bird or rodent control. Several pesticides can be used indoors to treat mite problems. Sprays and aerosols containing synergized pyrethrins should kill mites immediately on contact, though the treatment will only remain effective for up to a few hours.
- ✓ Insecticide sprays containing permethrin or bifenthrin are effective against many mites and should retain their killing properties for several weeks. Read the label carefully before spraying to make sure these products allow application to living areas, attics and crawl spaces. Indoor sprays should be applied only to the bases of walls and other potential entry points, not to furniture or other surfaces where people come into direct contact.

- ✓ When a nest can be located, it's best to first treat the area around the nest (e.g., the soffit or vent from which a bird nest is removed) with a pesticide, or else dust the area with a desiccant dust, such as diatomaceous earth. This should reduce the risk of live mites dispersing from the site and entering the indoor areas of the structure after the nest is removed.
- ✓ Long sleeves, gloves, and a tight-fitting dust mask are recommended when removing old bird or rodent nests to reduce the risk of exposure to ecto-parasites, like mites, and other pathogens.

Mites Medically Important to Humans

Mites are tiny eight-legged animals that are closely related to ticks. More than 30,000 species of mite have been identified. Even though they are very small, mites are important organisms to humans and other animals. Most species of mite are *beneficial decomposers* breaking down organic matter, allowing nutrients to be used by plants again. Most mites feed on organic matter, some mite species feed on plants while others have animal hosts. It is important to realize that mites that feed on plants, (spider mites, for example), cannot successfully feed and reproduce on human beings or other animals. Most mites that are animal parasites are fairly *host specific* only developing successfully on one species or group of animals that are closely related.

There are only a few mite species that cause medical problems for human beings.

House dust mite - One mite affecting some people is the house dust mite that causes allergic reactions in sensitive people. Allergic reactions are similar to a person having hay fever runny nose and eyes, and frequent sneezing. Some very sensitive individuals may have asthmatic reactions.

Scabies mite - A scabies infestation is caused by a tiny mite (1/50-inch) that burrows into the skin, feeds on cell liquids and lays 10-25 eggs along a horizontal burrow. Three to four days after hatching, the larval mites emerge from the skin, travel to another area where they burrow under the skin and repeat the process. Many animals, both domestic and wild, can be similarly infected by other species of scabies mite.

Probable questions:

1. Discuss the general morphology of ticks?
2. Write short note on tick's mouthparts?
3. Differentiate between hard ticks and soft ticks.
4. Describe the life cycle of tick with proper diagram.
5. Write down the mode of transmission of tick born bacterial disease.
6. Write down the symptoms, treatment, mode of transmission and causative agent of tick born bacterial disease.
7. Discuss the mode of transmission of relapsing fever.
8. Name the causative agent of typus.
9. Name virus borne disease and their causative agent.
10. Discuss general morphology of mites.
11. Write short notes on Chigger.
12. What is the causative agents of scabies? Describe the life cycle of that agent.
13. Discuss the different types of scabies.
14. How ticks and mites population can be controlled?

Suggested readings:

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6. Snodgrass, R.E. Principles of Insect Morphology. Cornell Univ. Press, USA
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Unit IX

Life cycle, mode of transmission, control and importance of Sand flies

Objective: In this unit you will know about Life cycle, mode of transmission, control and importance of Sand flies

Introduction

Sandfly (or sand fly) is a colloquial name for any species or genus of flying, biting, blood-sucking dipteran (fly) encountered in sandy areas. Sand fly may refer to members of the subfamily Phlebotominae within the Psychodidae. Some sand fly genera of the subfamily Phlebotominae are the primary vectors of leishmaniasis and pappataci fever. In the New World, leishmaniasis is spread by sand flies of the genus *Lutzomyia*; in the Old World, the disease is spread by sandflies of the genus *Phlebotomus*. Belize and Honduras are notorious in the Caribbean for their sand fly populations and travel pages frequently warn tourists to bring bug spray containing high concentrations of DEET.

Systematic Position

Kingdom-	Animalia
Phylum-	Arthropoda
Class-	Insecta
Order-	Diptera
Family-	Psychodidae
Subfamily-	Phlebotominae
Genus-	*Phlebotomous*

Several species of Sandflies are recognised. These are *P. papatasii*, *P. minutus*, *P. argentipes*, *P. orientalis*, *P. sergenti*, *P. nogouchi* etc. Among these species—*P. papatasii* is found in North India, *P. argentipes* in Assam and West Bengal, and *P. minutus* in other states of India.

Distribution

Old world phlebotomines are savanna and desert species (low rainfall)
New world phlebotomines are mainly inhabitants of forests (high rain fall)

Breeding place places

Sandflies breed in safe places which are rich in organic matter e.g. cracks, rodent burrows, animal shelters and privies e.g. dark, cool, humid niches like cracks, crevices of soil, manure, rocks, tree hollows, nests, tree crotches, animal burrows, (e.g. burrow of rodent), stables, livestock pens, well or any moist places. Larvae of sandflies are found in mud, sand and debris around the ends of the ponds, creeks springs, lakes, in tree holes or in slimy covered bark. They swim freely in water and they are normally found in twigs and leave trash. The larvae pupate on floating debris.

Host Range

Mostly broad host range

- *L. gomezi*: Feed on Birds and mammals
- *L. vespertilionis*: Bats
- *P. papatasi* : man and dog
- *P. argentipes*: man and cattle

Behaviour

These are blood sucking flies and they belong to the genus *Phlebotomus*. These are small, moth like flies, rarely over 5 mm long. Their bodies and wings are black and hairy. Female sand flies are blood feeders, but the males do not feed on blood. Females must consume a blood meal before they are able to develop eggs. However, both males and females also consume sugar-related nutrients that come from plant nectar or honeydew. Sand fly hosts vary a great deal. Some species feed on both mammals and reptiles, while *Lutzomyia shannani*, a common sand fly species in Florida and other coastal states feed on white-tailed deer, horses, donkeys, mules, cattle, swine, raccoons, rodents, birds and humans.
In general, sand fly bites are very painful. Most flies that bite humans feed during the evening and throughout the night. In some cases, flies will attack in the daytime, if they are disturbed while resting. Daytime resting sites include cavities close to the ground such as dry tree holes, hollow logs, palm tree crowns and the canopy of tropical and sub-tropical rain forest jungles. Another commonly found place for daytime resting is inside the home.

Morphology

Sand flies are small insects. Their length, including in females the head, thorax and abdomen and in males the coxites, is 1.2-3.7 mm. Head, thorax, abdomen and appendages are covered with hairs which give a "fluffy" appearance. The body of sand fly consists of three parts (Fig 1):

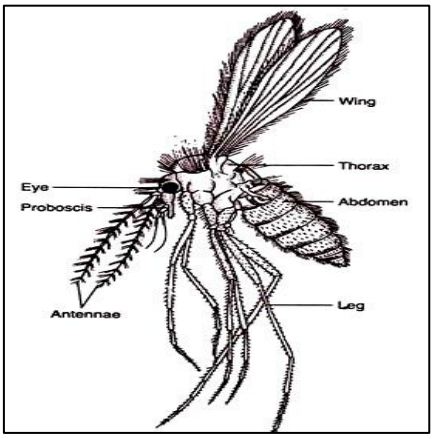

Fig 1: *Phlebotomus argentipes*

Head: The head bear a pair of long and hairy antenna. Palpi and proboscis and one pair of prominent black eyes are present. The pedicel of the males' antennae houses the Johnston's organ. The mouthparts are well-developed with cutting teeth on elongated mandibles in the proboscis, adapted for blood-sucking in females, but not in males.

Thorax: The thorax extends slightly over the head. It bears a pair of wings and three pair of legs. The wings are "V" shaped, upright in shape and hairy. The 2nd longitudinal vein is branched twice. The legs are long and slender and out of proportion to the size of the body.

Abdomen: The abdomen has ten segment and is covered with hairs. In the female the tip of abdomen is rounded while in male claspers are attached to last abdominal segment.

Life cycle of sand fly

The life cycle of sand fly is characterized by complete metamorphosis, having four developmental stages: egg, larvae (grub), pupae (cocoon) and adult. Sand flies complete their life cycle within 1-3 months, depending on the sand fly species and their environmental conditions (Fig 2).

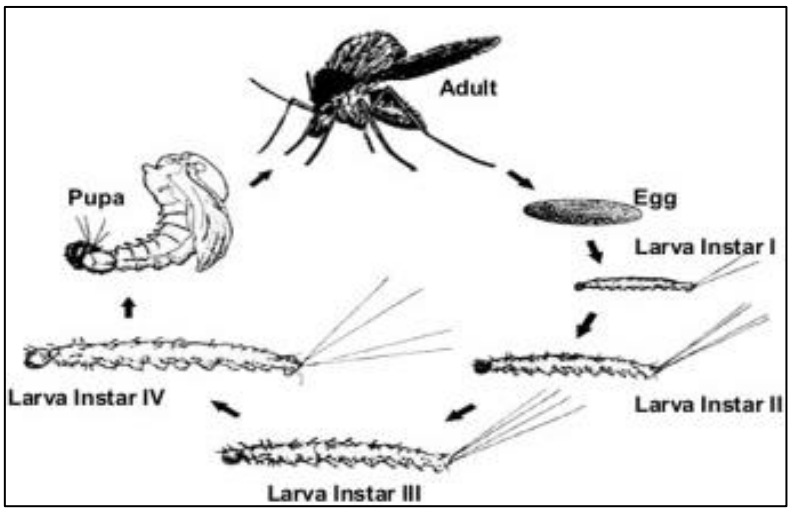

Fig 2: Life cycle of sand fly

Egg: The female generally lays eggs in the damp dark places in the cattle sheds & poultry. The eggs vary from 40-60 in number, brownish in colour and are laid singularly in small batches on moist surfaces like soil, in protected areas with high humidity and high organic matter. The eggs measure about 0.4 mm in length. They are elongated oval-shaped, pale at first and darkening on exposure to air with a single black "eye spot". Eggs hatch in 1-2 weeks.

Larva: The larva is maggot like structure, having large head, thorax and abdomen and two long bristle on last abdominal segment. Larva feed on decaying organic matter and become a pupa in about **2** weeks. First instar larva can be distinguished by the presence of two caudal bristles, all subsequent instars larvae are progressively larger than first-stage larvae bearing four caudal bristles. Fourth instar larvae also have a prominent sclerite on the dorsum of the penultimate segment. Before entering the pupal stage, the larvae stop feeding on the organic matter in their habitat and seek out a pupation site that is drier than its larval habitat. The

Pupa: The pupae of sand flies are golden brown in colour, 3 mm long and club-shaped. The narrow part is curved and head and thorax are curved backwards. Pupae are affixed to the surface of the substrate in which they developed by the final larval exuvium. The pupa stage lasts for about 1 week.

Adult: Shortly before emergence from the pupal case the wings and eyes turn black. Male sand flies emerge about 24 h before females, allowing their external genitalia time to rotate 180° to the correct position for mating before females have emerged. The average life of a sand fly is about 2 weeks.

Autogeny

Autogeny i.e. the ability to produce eggs without a bloodmeal has been reported for some sand fly species and suspected for others. But it is less common than in mosquitoes. Autogeny may help a sand fly population to increase quickly. Thus bring a rapid onset of the maximum *Leishmania* transmission period. While it has not been demonstrated for the Mediterranean vectors.

Sand Flies as Vectors

Sand fly diseases are transmitted by the bite of infected female sand flies in many parts of the world. Some of the more important sand fly transmitted diseases include, Leishmaniasis, Sand-fly fever, Oroya fever or Carrions disease etc.

1. Leishmaniasis:

Kala-azar, also known as Dum-dum fever, is a serious oriental disease of man. It is found in India, China, Mediterranean countries and parts of Africa and South America. Its causative agent is a pathogenic flagellate, known as *Leishmania donovani* which is transmitted by the bite of small blood sucking sandflies called *Phlebotomus argentipes* (Fig 1)). *Leishmania* species undergo multiplication as promastigotes *Phlebotomus argentipes*, but they are injected into a vertebrate host when the sand fly feeds, and they undergo additional multiplication, as amastigotes, in a variety of tissues.

The species concerned are as follows:
Indian vector: *P. argentipes*; Chinese vectors: *P. chinensis*, and *P. sergenti*; Mediterranean vectors: *P. perniciosus* (Italy and Sicily); Tropical American vector: *P. intermedius*; East African vector: *P. martini*.

A. Life cycle in Man:

(a) Infection:
L. donovani is transmitted to man by the sand-fly, *Phlebotomus argentipes*. In case of this parasite sand fly acts as vector. The insect vector which has fed on some suitable fruit or plant juice after feeding on infected human blood meal, shows an enormous number of parasites in its buccal cavity and pharynx. When such a vector bites a man, it introduces the parasites in the skin wound by its proboscis. Some authors are of opinion that the Indian vector (Sand fly) does not bite but spreads infection by being crushed possibly by slapping.

(b) Multiplication:
The parasites administered by the vector into human body are the promastigote or leptomonad form. Some of them, entering the blood circulation directly become destroyed while those entering the cells of R. E. system (liver, bone marrow, lymph node, spleen) change into amastigote or leishmanial forms. Multiplication by binary fission goes on continuously till the cells become packed with the parasites. The host cell is thereby enlarged and eventually ruptures (50 to 200 or even more may be found embedded in the enlarged host cell) (Fig 3).

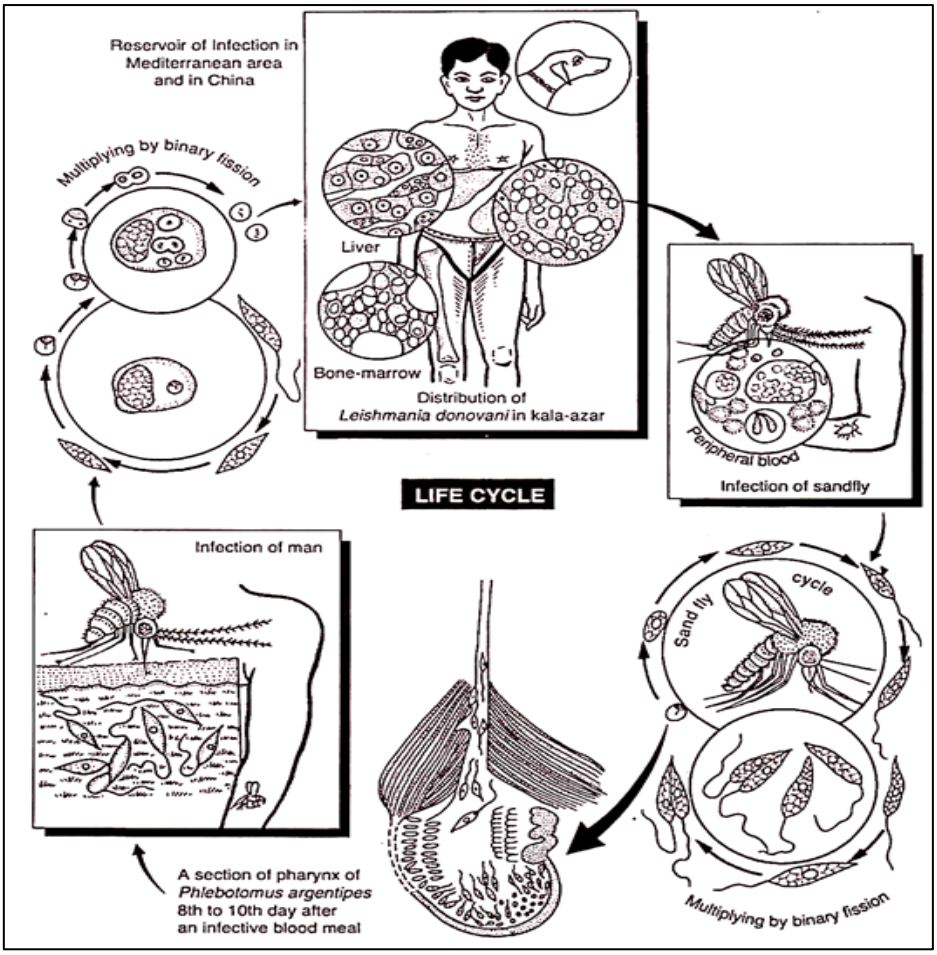

Fig 3: Life cycle of *Leishmania donovani* in man and sand fly

(c) Spread of infection:

When the number of parasites reaches upto 200 or even more, the host cell ruptures. The parasites liberated as a result of the rupture into the circulation are again either taken up by or invade fresh cells and the multiplication cycle is repeated so that the entire R. E. system becomes progressively infected. In the blood stream, some of the free amastigotes are phagocytosed by the neutrophil granulocytes and monocytes. A blood sucking insect draws these free amastigote forms as well as those within the monocyte during its blood meal. These heavily parasitized cells wander through the general blood circulation leading to a general infection.

B. Life cycle in vector (Sand fly):

(a) Transfer to vector:

When the vector sucks blood of an infected person, it receives free amastigotes as well as parasitized neutrophils and monocytes along with the blood meal.

(b) Development in the vector:

In the midgut of sand fly, the amastigote form becomes elongated and acquires a free flagellum, thus developing into promastigote form which again multiplies by binary fission producing an enormous number of flagellates. This multiplication proceeds in the midgut of this insect vector and the flagellates tend to spread forwards to the anterior part of digestive tract of (pharynx and buccal cavity) sand fly. A heavy pharyngeal infection of this insect vector is usually found between 6 to 9 days of its infective blood meal. This type of development is called anterior station development. The trans-

mission into a new host is thereby effected when such a heavily infested sand-fly bites the host (but salivary glands are not infected).

Types of leishmaniasis

Leishmaniasis comes in three forms: cutaneous, visceral, and mucocutaneous. Different species of the *Leishmania* parasite are associated with each form. Experts believe that there are about 20 *Leishmania* species that can transmit the disease to humans.

I. Cutaneous leishmaniasis

Cutaneous leishmaniasis causes ulcers on your skin. It's the most common form of leishmaniasis. Treatment may not always be necessary depending on the person, but it can speed healing and prevent complications.

II. Mucocutaneous leishmaniasis

A rare form of the disease, mucocutaneous leishmaniasis is caused by the cutaneous form of the parasite and can occur several months after skin ulcers heal. With this type of leishmaniasis, the parasites spread to your nose, throat, and mouth. This can lead to partial or complete destruction of the mucous membranes in those areas. Although mucocutaneous leishmaniasis is usually considered a subset of cutaneous leishmaniasis, it's more serious. It doesn't heal on its own and always requires treatment.

III. Visceral leishmaniasis

Visceral leishmaniasis is sometimes known as systemic leishmaniasis or kala azar. It usually occurs two to eight months after being bitten by a sand fly. It damages internal organs, such as your spleen and liver. It also affects your bone marrow, as well as your immune system through damage to these organs. The condition is almost always fatal if it's not treated.

Risk for leishmaniasis

Geography

The disease is found everywhere in the world except Australia and Antarctica. However, about 95 percent of cutaneous cases occur in: Americas, Central Asia, the Mediterranean basin, the Middle East. In 2015 over 90% of visceral cases occurred in: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan.

Socioeconomic conditions

According to the World Health Organization (WHO), poverty is a determining factor for the disease. In addition, leishmaniasis often occurs in areas where the following conditions are common: malnutrition, famine, lack of financial resources, large migrations of people caused by urbanization, emergency situations, war, environmental changes and climate change

Other infections

People who have weakened immune systems are at increased risk of this condition. HIV can influence the transmission of leishmaniasis and increase the risk of visceral leishmaniasis. HIV and leishmaniasis affect similar cells of the immune system. People infected with HIV are also often infected with leishmaniasis. In areas of Ethiopia, it's estimated that as many as 35% of people with leishmaniasis have HIV too.

Symptoms of leishmaniasis

People can carry some species of *Leishmania* for long periods without becoming ill. Symptoms depend on the form of the disease.

- **Cutaneous leishmaniasis**

The main symptom of this condition is painless skin ulcers. Cutaneous symptoms may appear a few weeks after being bitten by an infected sand fly. However, sometimes symptoms won't appear for months or years.

- **Mucocutaneous leishmaniasis**

In people with the mucocutaneous form of the disease, symptoms usually appear one to five years after the skin lesions. These are primarily ulcers in their mouth and nose or on their lips. Other symptoms may include: runny or stuffy nose, nosebleeds, difficulty breathing

- **Visceral leishmaniasis**

Symptoms often don't appear for months after the bite with this type of leishmaniasis. Most cases are apparent two to six months after the infection occurred. Common signs and symptoms include: weight loss, weakness, fever that lasts for weeks or months, enlarged spleen, enlarged liver, decreased production of blood cells, other infections, swollen lymph nodes. It is followed by general weakness, emaciation, anaemia and a peculiar darkening of the skin.

Diagnosis of leishmaniasis

It's important to tell your doctor if you lived in or visited a place where leishmaniasis is common. That way your doctor will know to test you for the parasite. If you have leishmaniasis, your doctor will use other tests to determine which species of *Leishmania* is the cause.

- **Cutaneous and Mucocutaneous leishmaniasis**

Your doctor may take a small amount of skin for a biopsy by scraping one of the ulcers. They'll often look for the DNA, or genetic material, of the parasite. They can use a variety of methods to identify the species of parasite causing the infection.

- **Visceral leishmaniasis**

Many times, people don't remember a bite from a sand fly. This can make the condition hard to diagnose. A history of living or traveling to an area of leishmaniasis is helpful. Your doctor may first perform a physical exam to look for an enlarged spleen or liver. They may then perform a bone marrow biopsy or take a blood sample for examination. A variety of specialized tests aid with diagnosis. Special chemical stains of bone marrow can help identify immune cells infected with the parasite.

Treatments for leishmaniasis

Antiparasitic drugs, such as amphotericin B (Ambisome), treat this condition. Your doctor may recommend other treatments based on the type of leishmaniasis you have.

- **Cutaneous leishmaniasis**

Cutaneous ulcers will often heal without treatment. However, treatment can speed healing, reduce scarring, and decrease risk of further disease. Any skin ulcers that cause disfigurement may require plastic surgery.

- **Mucocutaneous leishmaniasis**

These lesions don't heal naturally. They always require treatment. Liposomal amphotericin B and paromomycin can treat mucocutaneous leishmaniasis.

- **Visceral leishmaniasis**

Visceral disease always requires treatment. Several medications are available. Commonly used medicines include sodium stibogluconate (Pentostam), amphotericin B, paromomycin, and miltefosine (Impavido).

Prevention of leishmaniasis

There's no vaccine or prophylactic medication available. The only way to prevent leishmaniasis is to avoid getting bitten by a sand fly.

Follow these steps to help prevent being bitten by a sand fly:

- Wear clothing that covers as much skin as possible. Long pants, long-sleeved shirts tucked into pants, and high socks are recommended.
- Use insect repellent on any exposed skin and on the ends of your pants and sleeves. The most effective insect repellents contain DEET.
- Spray indoor sleeping areas with insecticide.
- Sleep on the higher floors of a building. The insects are poor fliers.
- Avoid the outdoors between dusk and dawn. This is when sand flies are most active.
- Use screens and air conditioning indoors when possible. Using fans might make it more difficult for the insects to fly.
- Use a bed net tucked into your mattress. Sand flies are much smaller than mosquitos, so you need a tightly woven net. Spray the net with insecticide containing pyrethroid if possible.

2. Sand-fly fever:

It is a mild virus disease and in many respects this Sand-fly fever resembles dengue. It comes on suddenly with fever, headache, pain in the eyes, stiffness of neck and back and rheumatic pain and is often followed by a prolonged period of malaise and depression.

It was experimentally shown by Doerr (1908) to be transmitted by *P. papatasii*. The insects become infective about 6 or 7 days after feeding on a patient in the first or second day of the fever. Since Sand flies are so short lived and frequently suck blood only once and since the disease appears as soon as the adults emerge in May in Mediterranean area, evidently being passed the winter in the larvae, trans-ovarial transmission had long been suspected.

Since only man is known to be susceptible, transovarial transmission seems necessary for survival of the virus from one season to another. Thus *P. papatasii* is the only known transmitter throughout the definite range of the disease.

3. Oroya fever or Carrions disease:

It is an acute febrile disease caused by a very minute organism, *Bartonella bacilliformis*. *P. nogouchi* is a Sand-fly which is found in South America. This Sand-fly acts as a vector of this pathogen which is responsible for Carrions disease.

Both the sexes of this Sand-fly suck blood of man and domesticated animals. The acute stage of the disease is charac-terised by high fever, severe anaemia, aches and albuminuria and is often fatal. In chronic cases it is followed by an eruption of nodules called verruga peruviana.

Prevention of sand flies

Insect repellent should be used on skin, so as to prevent the sand fly transmitted diseases. The use of mosquito netting sprayed with permethrin is recommended. Good sanitation and housekeeping prevents sand flies from lodging in the house. Insecticide can be sprayed on outdoor breeding sites.

Control

- Households are sprayed with residual insecticide on surfaces. Insecticide lindane has been proved effective. Spraying should be done in the human dwellings, cattle sheds and poultry. Its residue may remain effective for a period of 3 months. This has been the most effective method used. This control technique is also used for killing
- Destroy active qualities of reservoir species. Certain species of mammals can act as important reservoirs of *Leishmania.* By depriving life to reservoir species which live near human households, disease rates can be decreased. One can make a use of rodenticide.
- Sanitation: Removal of shrubs and vegetation, filling of cracks and crevices in the wall & floor and distance of cattle sheds and poultry from human habitations.

Importance of Sand fly

Sand flies are biting insects that share some similarities with mosquitoes, being classified in the suborder, the Nematocera. They are widely spread in regions with warm temperate through subtropical to tropical climates. The veterinary and medical importance of sand flies results from their ability to transmit viral, bacterial and protozoal diseases.

Sand flies belong to the insect order Diptera, suborder Nematocera. Within this suborder the family Psychodidae includes biting sand flies in diverse genera and non-biting owl-midges or moth flies (genus Psychoda). The subfamily *Phlebotominae* consists of about 700 phlebotomine species. Among the existing phlebotomine genera two have been proven as vectors of one of the main zoonotic pathogens worldwide, the protozoan parasite *Leishmania*. These are *Phlebotomus* in the Old World and *Lutzomyia* in the New World.

Sand fly-transmitted diseases are a major health issue for dogs. Even with preventative measures, mosquito control with repellent and insecticidal efficacy is crucial.

Probable questions:

1. Write down the systematic position of sand fly.
2. Name the sand fly species which use man as their exclusive host?
3. Describe the morphology of adult sand fly with diagram.
4. Describe the life cycle of sand fly with diagram.
5. Name the causative agent of leishmaniasis.
6. Discuss the life cycle of *Leishmania* with diagram.
7. Describe the symptoms of different types of leishmaniasis.
8. Describe the preventive measures of leishmaniasis.
9. Write short notes on sand fly fever.
10. Describe the importance of sand fly.

Suggested readings:

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Unit X

Life cycle, mode of transmission, control and importance of Tabanid fly

Objective: In this unit you will know about Life cycle, mode of transmission, control and importance of Tabanid fly

Introduction

Deer flies, horse flies and yellow flies, collectively called tabanids, are a large diverse group of blood feeding flies. Their mouthparts are stout, blade-like and they inflict a painful bite when feeding. Because of the painful bite, they are frequently interrupted when feeding; about 10% are successful on the first attempt. The most important pest species in this group belong to one of three genera: *Chrysops*, *Hybomitra* and *Tabanus*.

Deer flies range in size from ¼" to ½", have coloured eyes and banding on the wings. They readily attack humans, companion animals, livestock and wildlife. Horse flies are larger (½" to >1") with banded eyes and clear wings. They are serious pest of livestock and wildlife and seldom attack humans.

Horse flies are in the genus *Tabanus* and deer flies are in the genus *Chrysops*.

The yellow fly, *Diachlorus ferrugatus* (Fabricius), is known in Florida as a fierce biter. Like mosquitoes, it is the female fly that is responsible for inflicting a bite. The males are mainly pollen and nectar feeders. Tabanids are most likely encountered in hot summer and early fall weather. They are active during daylight hours.

Tabanids use a variety of aquatic habitats (marshes, creek and pond margins, in and along streams for egg-laying and development. Eggs are commonly deposited on vegetation overhanging habitats that contain mud and saturated vegetation. The eggs hatch and the larvae develop in the wet environment. The larvae are predaceous and cannibalistic. Some require up to three years to complete development.

Tabanid fly (*Tabanus* spp.)

Common name – Horse fly

Horse-flies or horseflies are true flies in the family Tabanidae in the insect order Diptera. They are often large and agile in flight, and the females bite animals, including humans, to obtain blood. They prefer to fly in sunlight, avoiding dark and shady areas, and are inactive at night. They are found all over the world except for some islands and the Polar Regions.

Distribution

Horse flies and deer flies are world-wide in distribution. They are, however, unreported in Hawaii, Greenland, and Iceland. In the United States, Florida produces a large population of tabanids because of the availability of suitable habitat. Florida's mild climate and large permanently wet and undeveloped areas provide good breeding areas.

Morphology

Eggs: Eggs are laid in masses ranging from 100 to 1000 eggs. Eggs are laid in layers on a vertical surface such as overhanging foliage, projecting rocks, sticks and aquatic vegetation (Fig 1). Aquatic

vegetation is preferred. A shiny or chalky secretion, which aids in water protection, often covers eggs. The vertical surfaces on which the eggs are deposited are always directly over water and wet ground favourable to the development of larvae. The female will not deposit egg masses on vegetation that is too dense. Eggs are initially a creamy white color but soon darken to gray and black. Eggs are cylindrical in shape and measure from 1 to 2.5 mm in length. Eggs hatch in five to seven days, depending upon ambient weather conditions, and the larvae fall to the moist soil and water below.

Fig 1. Deer fly, *Chrysops* sp., egg mass after darkening.

Larvae: Larvae use a hatching spine to break out of the egg case (Fig 2). The larvae are aquatic, semi-aquatic or terrestrial. *Chrysops* spp. are termed "hydrobionts" and are found in areas with high water content. *Tabanus* spp. prefer drier substrates and are "hemi-hydrobionts". The larvae taper at each end and are usually whitish in color, but also can be brownish or green depending on the species. Black bands are found around each segment of the body in many species. The larva breathes through a tracheal siphon located at their posterior end. The larva has a small head and 11 to 12 additional segments. Larvae pass through six to nine stadia. The time spent in the larval stage can last from a few months to a year. The larvae of *Chrysops* feed upon organic matter in the soil. *Tabanus* spp. feed upon insect larvae, crustaceans, and earthworms. Even though the *Tabanus* spp. are considered to be carnivorous and cannibalistic, reports of as many as 120 larvae per square yard have been found. The larva moves into the upper 2.5 to 5.0 cm of the soil, where it is drier, when it is ready to pupate. Within two days after moving to the surface the pupal stage is reached.

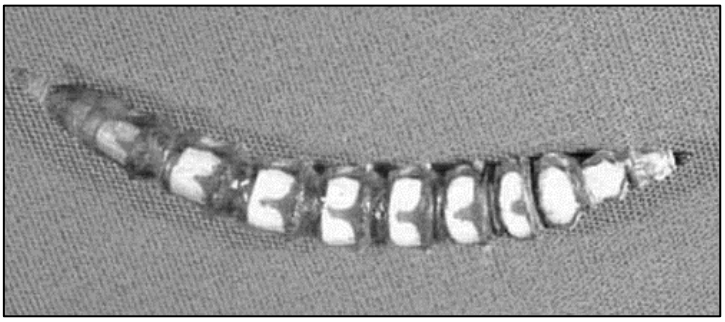

Fig 2. Typical larva of a Tabanidae species

Pupae: The pupae are brown colored, rounded anteriorly, tapered posteriorly, and have leg and wing cases attached to the body (Fig 3). There is a row of spines encircling each abdominal segment. A pupal "aster" consisting of six pointed projections is located at the apex of the abdomen. The pupal stage generally lasts from two to three weeks.

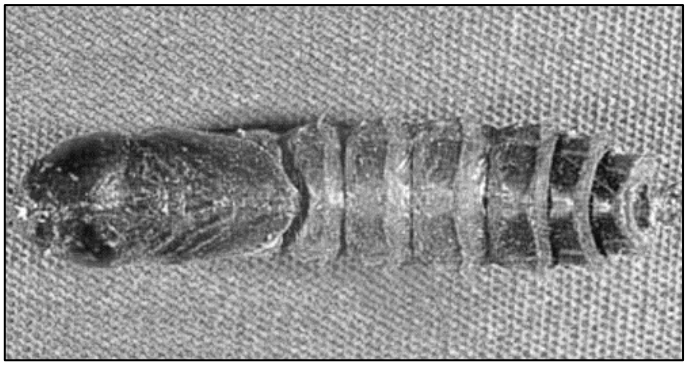

Fig 3. Typical Tabanidae pupa

Adult: The adult fly emerges from the pupal case via a slit located along the thorax of the case (Fig 4, 5, 6). In most species the males emerge before the females. After emergence of both sexes, the flies mate. Mating starts with the male pursuing the female. Mating is initiated in the air and completed on the ground. The female then deposits an egg mass and is ready to seek a host. Adult Tabanidae are large flies with broad bodies and bulging eyes. The males are easily differentiated from female flies because eyes are contiguous in the males and widely separated in the females. The antennae are three segmented. The thorax and abdomen are covered with fine hairs. Deer flies range in length from 7 to 10 mm while horse flies are from 10 to 25 mm. The deer flies are yellow to black, have stripes on the abdomen, and possess mottled wings with dark patches. Yellow flies are yellowish with the same body shape of deer flies, but have dark purple to black eyes marked with florescent green lines. Horse flies are black to dark brown with green or black eyes. Adult deer flies have apical spurs on the hind tibiae that are not present in horse flies.

Fig 4. Adult female deer fly, *Chrysops pikei*

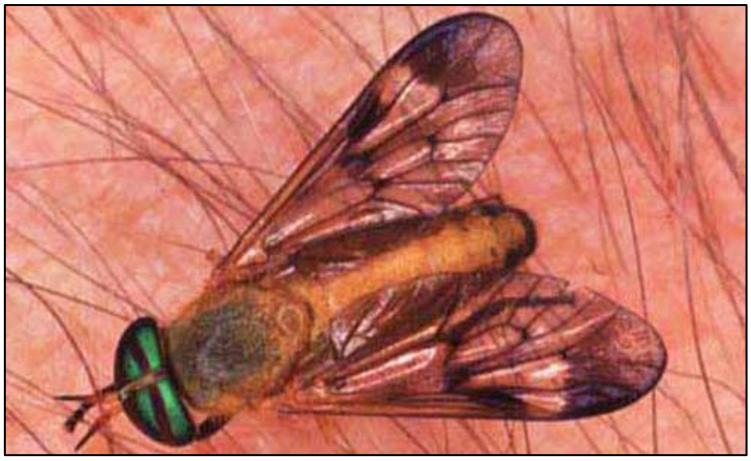

Fig 5**.** Adult yellow fly, *Diachlorus ferrugatus* (Fabricius)

Fig 6: Adult horse fly, *Tabanus* sp.

Biology

Diet and biting behaviour: Adult horse-flies feed on nectar and plant exudates, and some are important pollinators of certain specialised flowers. Both males and females engage in nectar feeding, but in addition to this, females of most species are anautogenous, meaning they require a blood meal before they are able to reproduce effectively. To obtain the blood, the females bite animals, including humans, while the males are harmless. It takes the female about six days to fully digest its blood meal and after that it needs to find another host. It seems that the flies are attracted to a potential victim by its movement, warmth, and surface texture, and by the carbon dioxide it breathes out. The flies mainly choose large mammals such as cattle, horses, camels, and deer, but few are species specific. They have also been observed feeding on smaller mammals, birds, lizards and turtles, and even on animals that have recently died. Because their bite is irritating to the victim, they are often brushed off, and may have to visit multiple hosts to obtain sufficient blood. This behaviour means that they may carry disease-causing organisms from one host to another.

The mouthparts of females are of the usual Dipteran form and consist of a bundle of six chitinous stylets that, together with a fold of the flesh labium, form the proboscis. On either side of these are two maxillary palps. When the insect lands on an animal it grips the surface with its clawed feet, the labium is retracted, the head is thrust downwards and the stylets slice into the flesh. Some of these have sawing edges and muscles can move them from side-to-side to enlarge the wound. Saliva

containing anticoagulant is injected into the wound to prevent clotting. The blood that flows from the wound is lapped up by another mouthpart which functions as a sponge. Horsefly bites can be painful for a day or more; fly saliva may provoke allergic reactions such as hives and difficulty with breathing. Tabanid bites can make life outdoors unpleasant for humans, and can reduce milk output in cattle. They are attracted by reflections from water which are polarized, making them a particular nuisance near swimming pools. Since tabanids prefer to be in sunshine, they normally avoid shaded places such as barns, and are inactive at night.

Attack patterns vary with species: Large species of *Tabanus* buzz loudly, fly low, and bite ankles, legs or backs of knees; *Chrysops* fly somewhat higher, bite the back of the neck, and have a high buzzing note. It has been suggested that the striped hides of zebras have evolved to reduce their attractiveness to horse-flies and tsetse flies than either plain dark or plain white hides. The closer together the stripes, the fewer flies are visually attracted; the zebra's legs have particularly fine striping, and this is the shaded part of the body that is most likely to be bitten in other, unstriped equids. This does not preclude the possible use of stripes for other purposes such as signaling or camouflage.

Life Cycle

Mating often occurs in swarms, generally at landmarks such as hilltops. The season and time of day, and type of landmark, used for mating swarms is specific to particular species (Fig 7).

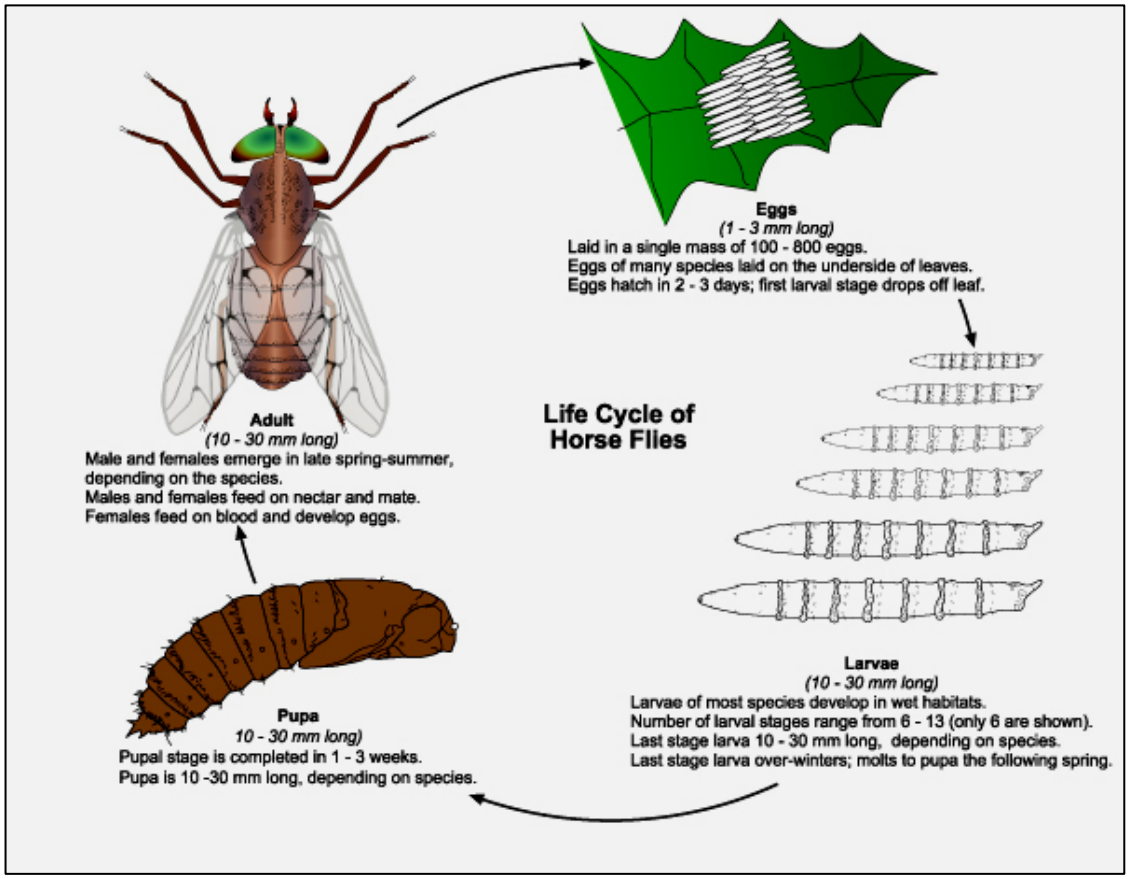

Fig 7: Life cycle of horse fly

Eggs are laid on stones or vegetation near water, in clusters of up to one thousand, especially on emergent water plants. The eggs are white at first but darken with age. They hatch after about six days, the emerging larvae using a special hatching spike to open the egg case. The larvae fall into the

water or onto the moist ground below. *Chrysops* species develop in particularly wet locations while *Tabanus* species prefer drier places. The larvae are legless grubs, tapering at both ends. They have small heads and eleven or twelve segments and moult six to thirteen times over the course of up to a year or more. In temperate species, the larvae have a quiescent period during winter (diapause) while tropical species breed several times a year. In the majority of species they are white, but in some, they are greenish or brownish, and they often have dark bands on each segment. A respiratory siphon at the hind end allows the larvae to obtain air when submerged in water. Larvae of nearly all species are carnivorous, often cannibalistic in captivity, and consume worms, insect larvae, and arthropods. The larvae may be parasitized by nematodes, flies of the families Bombyliidae and Tachinidae; and Hymenoptera in the family Pteromalidae. When fully developed, the larvae move into drier soil near the surface of the ground to pupate.

The pupae are brown and glossy, rounded at the head end and tapering at the other end. Wing and limb buds can be seen and each abdominal segment is fringed with short spines. After about two weeks, metamorphosis is complete, the pupal case splits along the thorax and the adult fly emerges. Males usually appear first, but when both sexes have emerged, mating takes place, courtship starting in the air and finishing on the ground. The female needs to feed on blood before depositing her egg mass.

Damage

Tabanids lie in wait in shady areas under bushes and trees for a host to happen by. Sight is the main host finding mechanism, but carbon dioxide and odor also play a role. Moving objects, especially if dark colored, are most prone to attack. Attacks occur during daylight hours with a peak beginning at sunrise and lasting three hours. A second peak is two hours before sunset and commences shortly after. Attack frequency is low on overcast days or at temperatures below 22 and above 32 ºC. On livestock, biting occurs on the abdomen, legs, and neck. Tabanids inflict deep wounds that cause a flow of blood. The mandibles and maxillae penetrate the skin in a scissor-like action. Anticoagulants in the saliva are pumped into the wound and the blood is ingested through the sponging labella. Pathogens may be transmitted from flies that are disturbed while feeding on one animal and begin feeding on another. It is known that deer flies can mechanically vector Tularemia and Loa loa, and horse flies transmit Anthrax. Fly attacks result in lowered gains and low milk production in livestock animals. In 1976, estimated losses in the United States were at 40 million dollars. One cattle ranch in Kentucky lost an average 100 lbs. per animal due to tabanids. It is not uncommon to see as many as 100 flies feeding on an animal at one time. Twenty to thirty flies feeding for six hours are capable of taking 100 cc of blood.

Disease transmission

Certain characteristics of tabanids enhance their capabilities to transmit pathogens:

(1) anautogeny, the necessity of a blood meal for development of eggs, stimulating hostseeking behavior;

(2) telmophagy, through which blooddwelling pathogens can enter the pools from which flies suck blood;

(3) Relatively large blood meals, enhancing the possibility that pathogens will be imbibed;

(4) Long engorgement time, enabling pathogens to infect a fly’s tissues; and

(5) intermittent feeding behavior, increasing the chances for mechanical transmission of pathogens.

Tabanids are known vectors for some blood-borne bacterial, viral, protozoan and worm diseases of mammals, such as the equine infectious anaemia virus and various species of *Trypanosoma* which cause diseases in animals and humans.

Tabanids are involved in transmission of protozoan, helminthic, bacterial, and viral diseases of animals and humans. Among diseases caused by protozoa, two species of *Trypanosoma* are transmitted mechanically by tabanids. *Trypanosoma evansi*, the causative agent of surra in many wild and domestic animals, is spread by species of Tabanus. Tabanids are known to transmit anthrax among cattle and sheep, and tularemia between rabbits and humans. Other vectors, such as stable flies, other genera of horse flies, and vampire bats, also can be involved; but Tabanus spp. appear to be the most effective vectors of this trypanosome. *Trypanosoma theileri* is a cosmopolitan parasite of cattle and antelopes. Cyclopropagative development occurs in the insect gut, so the tabanid species involved are actually true intermediate hosts. Examples of such species are Haematopota pluvialis, Tabanus striatus, and *T. glaucopis*. The African eye worm, Loa loa (chapter 29), is transmitted by species of Chrysops. There appear to be two strains of L. loa, one in monkeys in the forest canopy and one in humans. Night-feeding *Chrysops langi* and *C. centurionis* transmit the former, and diurnal C. silaceus and *C. dimidiatus* transmit the latter.

Medical importance of Tabanidae is two sided: the annoyance and blood loss occasioned by the bite and infections transmitted mechanically and biologically by the flies. Because of the large size of mouthparts, tabanid bites are quite painful. Most people have little or no allergic reaction to them, although such sequellae can occur. Their annoyance factor may seriously interfere with use of recreational areas, and field-workers and timber-workers may have lowered productivity as a result of harassment by these flies.

A serious problem for livestock is blood loss, interrupted grazing, and energy consumed in trying to escape the insects. Tethered or caged animals particularly suffer from these flies because they are unable to escape their tormentors, even briefly. One well-known parasitologist reported seeing a caged mule deer simultaneously being fed on by a dozen or more *Tabanus punctifer* and dozens of open, freely bleeding wounds covering the wretched animal's face. No Tabanids are strictly host specific, but most have host preferences. Thus, *Haematopota*, with at least 300 species, feeds mainly on cattle and antelope, with which it may have evolved. Birds are attacked uncommonly; amphibians and reptiles may provide nourishment for these parasites.

Control

Mechanical: Malaise traps are most often used to capture them and these can be modified with the use of baits and attractants that include carbon dioxide or octenol. A dark shiny ball suspended below them that moves in the breeze can also attract them and forms a key part of a modified "Manitoba trap" that is used most often for trapping and sampling

Chemical: Cattle can be treated with pour-on pyrethroids which may repel the flies, and fitting them with insecticide impregnated eartags or collars has had some success in killing the insects.

Probable questions:

1. Write the scientific names of Tabanid flies.
2. Describe the morphology of Tabanid fly
3. Discuss the biology of Tabanid fly.
4. Describe the life cycle of horse fly with suitable diagram.
5. Discuss the nature of damage of Tabanid fly,
6. Write short note on disease transmission of Tabanid fly.

Suggested readings:

1. Bernays, E.A. and Chapman, R.F. (). Host Selection by Phytophagous insects. Chapman and Hall, New York, USA
2. Gullan, P.J. and Cranston, P.S. (). The Insects: An Outline of Entomology. Wiley Blackwell.
3. Hati, A.K. (2010). Medical Entomology. Allied Book Agency, Kolkata.
4. Lehane, M.J. (2005). The Biology of Blood Sucking Insects. 2nd Edn. Cambridge Univ Press.
5. Nation, J.L. Insect Physiology and Biochemistry. CRC Press, USA
6. Snodgrass, R.E. Principles of Insect Morphology. Cornell Univ. Press, USA
7. Wilson, E.O. The Insect Societies. Harvard Univ. Press, UK

Unit XI

Life cycle, mode of transmission, control and importance of Black fly

Objective: In this unit you will know about life cycle, mode of transmission, control and importance of black fly

Introduction

Black flies, known also as "buffalo gnats" and "turkey gnats," are very small, robust flies that are annoying biting pests of wildlife, livestock, poultry, and humans. Their blood-sucking habits also raise concerns about possible transmission of disease agents. You are encouraged to learn more about the biology of black flies so that you can be better informed about avoiding being bitten and about their public health risk.

Common name: black flies
Scientific name: *Simulium* spp

Distribution

Black flies are found in many parts of the US and Canada, including Florida. Populations in Florida normally are not present in numbers large enough to be noticed by humans. *Simulium slossonae* is found in Florida from Dade country north to Duval County, and west to Escambia County. In some areas it is present all year long. In South Carolina, population peaks have been recorded in late July, late September, and late October, but large numbers have been found in Florida between April and November. Overall, Florida has 18 different black fly species, the most common with populations present from August through July. Only female black flies feed on blood. Males feed mainly on nectar.

Black flies can occur in enormous numbers. Immature stages develop in oxygenated water sources, therefore adults are usually associated with slow moving streams, creeks, or rivers where the immature stages develop. Flowing water does not necessarily imply white water rapids, but water must be moving. Water in lakes and ponds that is not flowing is unsuitable for black fly development. *Simulium slossonae* prefers fairly small, slow moving streams with an average velocity of 1.5 ft per second. Water is often tea-colored, with ample vegetation, light shade, and a pH of 4.4 to 4.5. *Simulium slossonae* makes good use of temporary streams that flow seasonally.

Types of Black Flies

Black flies are true flies (Order Diptera) in the family Simuliidae, which includes more than 1,700 species worldwide. In North America, 255 species in 11 genera have been identified, but additional species remain to be discovered and named. Very little is known about black flies in Indiana, and there are no estimates of the number of species in the state. For perspective, 12 species have been documented in Illinois, while over 30 species have been documented in both Minnesota and Wisconsin, where black fly habitats are more abundant.

Morphology

Black flies range in size from 5 to 15 mm, and they are relatively robust, with an arched thoracic region (Fig 1). They have large compound eyes, short antennae, and a pair of large, fan-shaped wings. Most species have a black body, but yellow and even orange species exist.

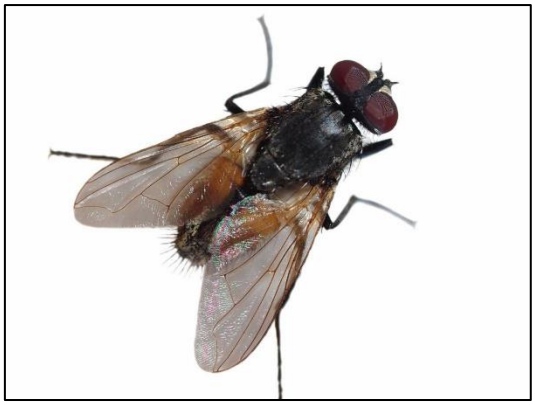

Fig 1: Adult black fly

Life Cycle

Black flies undergo a type of development known as "complete metamorphosis" (Fig 2). This means the last larval stage moults into a non-feeding pupal stage that eventually transforms into a winged adult.

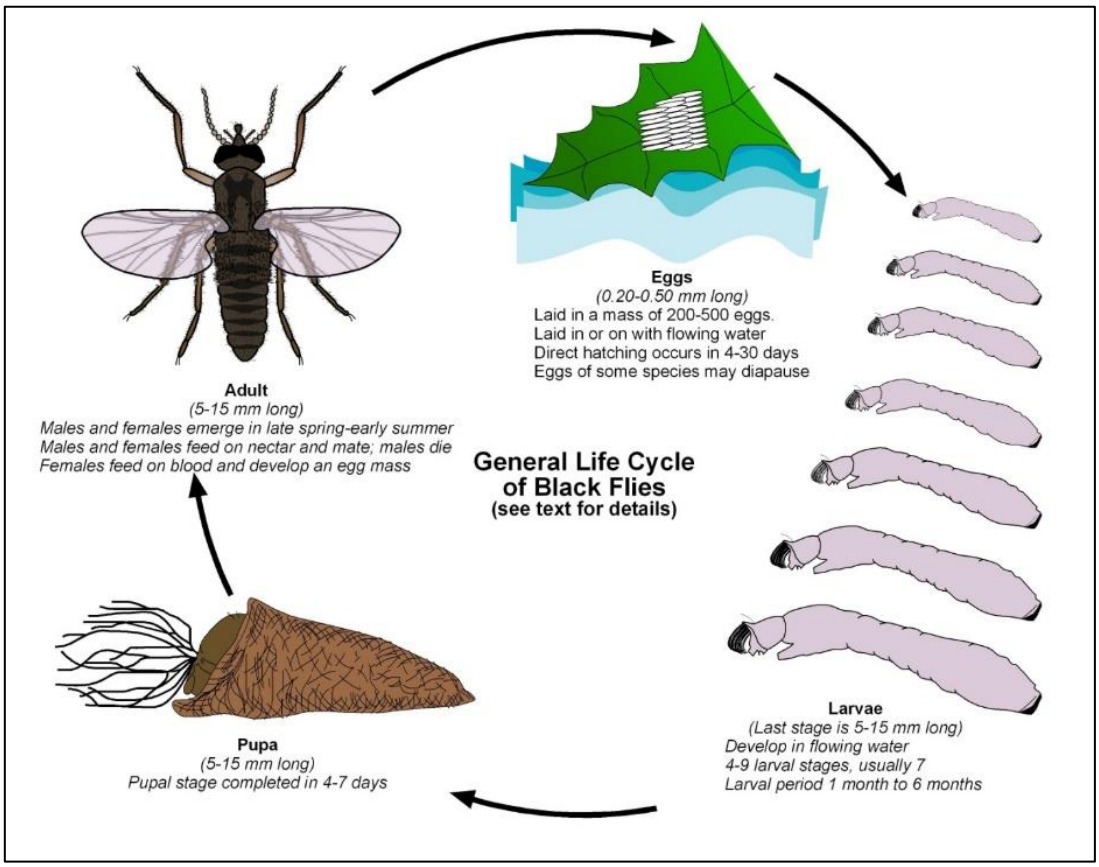

Fig 2: Black fly life cycle

Egg:

Adult black flies are small insects that measure 1 to 5 mm in length, and possess a shiny thorax (middle of the fly) that ranges in colour from black to various shades of grey or yellow. After taking a blood meal, females develop a single batch of 200-500 eggs. Most species lay their eggs in or on flowing water, but some attach them to wet surfaces such as blades of aquatic grasses. The length of

time it takes an egg to hatch varies greatly from species to species. Eggs of most species hatch in 4-30 days, but those of certain species may not hatch for a period of several months or longer.

Larva:

The number of larval stages ranges from 4-9, with 7 being the usual number. The duration of larval development ranges from 1-6 months, depending in part on water temperature and food supply. Larvae pass through six stages before reaching the pupal stage. The life cycle stage that passes though winter is the last stage larva attached underwater to rocks, driftwood, and concrete surfaces such as dams and sides of man-made channels.

Larvae remain attached to stationary objects in flowing water, held on by silken threads extruded from glands located at the end of the bulbous abdomen. Depending on species, mature larvae range from 5-15 mm in length and may be brown, green, gray, or nearly black in color. They possess a large head that bears two prominent structures known as "labral fans" that project forward (see Figure 2). Labral fans are the primary feeding structures, filtering organic matter or small invertebrates out of the water current.

Pupa:

The pupal stage is formed the following spring or summer, typically in the same site as the last stage larva, but may occur downstream following larval "drift" with the current. Adults emerge from the pupal stage in 4-7 days and can live for a few weeks. Adults of most species are active from mid-May to July. The number of generations completed in one year varies among species, with some having only one generation, but most species that are major pests complete several generations per year.

Pupae remain attached to stationary objects in flowing water as well. They typically are orange and appear mummy-like because the developing wings and legs are tightly attached to the body. Pupae of many species produce a delicate, silken "cocoon" of varying density, weave, and size that partially or nearly entirely encloses them; other species produce hardly any cocoon at all.

Black fly larvae and pupae develop in flowing water, typically non-polluted water with a high level of dissolved oxygen. Suitable aquatic habitats for black fly larval development vary greatly and include large rivers, icy mountain streams, trickling creeks, and waterfalls. Larvae of most species typically are found in only one of these habitats.

Adult:

Simulium slossonae adults may fly four to eight miles from breeding sites in search of hosts, then return after feeding to breed and lay eggs. In parts of Africa, adult female black flies may travel more than forty miles from aquatic breeding sites to find blood meals. So, the biting problem at a particular location may be generated at some distance away, even in Florida.

Feeding habits of adult black flies

It is estimated that females of 90% of the black fly species require a blood meal for the development of eggs. Those of most species feed on mammals, while others feed on birds. Females of some black fly species feed on only one host, whereas others are known to feed on over 30 different host species. No North American species feed exclusively on humans. Male black flies are not attracted to humans, and their mouthparts are not capable of biting.

Females of most species of black flies feed during the day, usually biting on the upper body and head. Unlike certain species of mosquitoes and biting midges, black flies do not enter human structures to seek blood meals.

Mode of transmission (onchocerciasis)

The parasites that cause onchocerciasis are transmitted from human to human through the bites of blackflies, which belong to *Simulum* species. Blackflies breed in fast-flowing rivers and streams, with good vegetation nearby. Unlike mosquitoes and sand flies, they bite during the day when people are active in the area.

The adult worms mate in the infected person, and the eggs hatch into microscopic worms called microfilaria, which burrow through the body tissues. The person's immune system attacks the microfilaria, causing inflammation and damage in the surrounding tissues. Sight defects and eventually blindness develops when the microfilaria are embedded in the person's eye. When a female blackfly bites an infected person during a blood meal, the microfilaria are transferred from the person to the fly. Over the course of one to three weeks, the microfilaria develop inside the blackfly to form infective larvae. These are then passed on to other people when the blackfly takes another blood meal. The microfilaria migrate to the skin, lymph nodes and eyes of the infected person, causing inflammation and tissue damage.

In the human host, the larvae migrate into the skin, and nodules (swellings) form around them. They slowly mature into adult worms, which can live for 15 years in the human body. After mating, the female worm releases around 1,000 microfilaria a day into the surrounding tissue. Microfilaria live for one to two years, moving around the body. When they die, they cause an inflammatory response which leads to the clinical manifestations and complications such as blindness.

Damage

Black flies have preferences for a wide range of individual host species. Adult females feed on the blood of humans, cattle, horses, sheep, goats, poultry, other livestock and wild mammals and birds. Each black fly species may prefer one type of host over another. The black fly common name sometimes indicates host specificity, for example the turkey gnat. Black flies are daytime biters preferring low wind conditions. They are not restricted to shaded or humid sites, and usually do not go indoors. They are attracted to hosts from a distance by smell, heat, and by sight. The female flies swarm around and crawl on the host preferring the head, hair, and ears as well as any skin that is exposed or that they can crawl onto. *Simulium slossonae* is primarily a bird feeder and probably preys on wild turkeys to some extent. It is the primary vector of the protozoan blood parasite, *Leucocytozoon smithi*, in Florida. This parasite is restricted only to birds, especially turkeys. *Simulium slossonae* will feed on domestic turkeys as well as chickens and other poultry. Several cases of chicken mortality attributed to black fly feeding were reported in Florida during the first three months of 1998.

Female black flies are blood feeders whose bites can itch and persist for several days. The flies bite by cutting into the skin and feeding on the pool of blood that forms in the hole they make. Anticoagulants injected into the feeding site by black flies can cause mild to severe allergic reactions in sensitive individuals. Strong reactions include fever, nausea and allergic dermatitis. Large black fly populations and strong bite reactions can be life threatening and have been reported to kill domestic animals. Black fly bites are very painful because of the hole that is cut in the skin, the anticoagulants and other materials that are injected, and the immunological differences between insect and hosts' tissues.

Some people are very attractive to black flies and have strong feeding reactions. Others appear to repel black flies and are bitten little if at all. Although *Simulium slossonae* is primarily a bird feeder, large swarms are attracted to people. Fortunately in Florida, most of the black flies attracted to people

do not bite. Anyone showing strong allergic reactions should consult a physician for immediate treatment. Treatments for bites include antipruritic lotions or creams.

Public Health importance

1. Nuisance

Large numbers flies can be bring significant nuisance by disturbing people during work and at leisure. Flies soil the inside and outside of houses with their faeces. They can also have a negative psychological impact because their presence is considered a sign of unhygienic conditions.

2. Diseases

Flies can spread diseases because they feed freely on human food and filthy matter

Flies pick up disease-causing organisms while crawling and feeding those that stick to the outside surfaces of the fly may survive for only a few hours, but those that are ingested with the food may survive in the fly's crop or gut for several days

(1) Annoyance (bite and persistence)
(2) "*Black Fly Fever"* – reaction to salivary secretions.
(3) *Onchocerciasis* "River Blindness"

Onchocerciasis, also known as river blindness, is a disease that affects the skin and eyes. It's caused by the worm *Onchocerca volvulus*. *Onchocerca volvulus* is a parasite. It's spread to humans and livestock through the bite of a type of blackfly from the genus *Simulium.* This type of blackfly is found near rivers and streams. That's where the name "river blindness" comes from.

Symptoms

There are different stages of onchocerciasis. In earlier stages, you may not have any symptoms. It can take up to a year for symptoms to appear and the infection to become apparent.

Once the infection becomes severe, symptoms may include:

- skin rashes
- extreme itching
- bumps under the skin
- loss of skin elasticity, which can make skin appear thin and brittle
- itching of the eyes
- changes to skin pigmentation
- enlarged groin
- cataracts
- light sensitivity
- loss of vision

Causes

You can develop river blindness if you're bitten repeatedly by infected female blackflies. The blackfly passes the larvae of the worm *Onchocercidae* through the bite. The larvae move to the subcutaneous tissue of your skin, and mature into adult worms over 6 to 12 months. The cycle repeats when a female blackfly bites a person infected with onchocerciasis and ingests the parasite.

Adult worms can live for 10 to 15 years and may produce millions of microfilariae during that time. Microfilariae are baby or larval worms. Symptoms appear when microfilariae die, so symptoms can continue to worsen the longer you are infected. The most extreme, longest-lasting cases result in blindness.

Risk factors

You're at increased risk for onchocerciasis if you live near fast-running streams or rivers in intertropical areas. That's because blackflies live and breed in these areas. Ninety percent of cases are in Africa, but cases have also been identified in Yemen and in six countries in Latin America. It's unusual for casual travelers to contract the disease because repeated bites are necessary for the infection to be transmitted. Residents, volunteers, and missionaries in areas of Africa are at the greatest risk.

Diagnosis

There are several tests used to diagnose onchocerciasis. Usually, the first step is for a clinician to feel the skin to try to identify nodules. Your doctor will do a skin biopsy, known as a skin snip. During this procedure, they'll remove a 2- to 5-milligram sample of the skin. The biopsy is then placed in a saline solution, which causes the larvae to emerge. Multiple snips, usually six, are taken from different parts of the body.

An alternative test is called the Mazzotti test. This test is a skin patch test using the drug diethylcarbamazine (DEC). DEC causes the microfilariae to die rapidly, which can lead to severe symptoms. There are two ways that clinicians may use DEC to test for onchocerciasis. One way is by giving you an oral dose of the medication. If you're infected, this should cause severe itching within two hours. The other method involves putting DEC on a skin patch. That will cause localized itching and a rash in people with river blindness.

A more rarely used test is the nodulectomy. This test involves surgically removing a nodule and then examining it for worms. An enzyme-linked immunosorbent assay (ELISA) test can also be performed, but it requires expensive equipment.

Two newer tests, polymerase chain reaction (PCR) and rapid-format antibody card tests, show promise.

PCR is highly sensitive, so it only requires a small skin sample — about the size of a small scratch — to perform the test. It works by amplifying the DNA of the larvae. It's sensitive enough that even very low-level infections can be detected. The drawback to this test is cost.

The rapid-format antibody card test requires a drop of blood on a specialized card. The card changes color if antibodies to the infection are detected. Because it requires minimal equipment, this test is very useful in the field, meaning you don't need access to a lab. This type of test is being widely used and efforts are underway to standardize it.

Treatment

The most widely used treatment for onchocerciasis is ivermectin (Stromectol). It's considered safe for most people and only has to be taken once or twice a year to be effective. It also doesn't require refrigeration. It works by preventing the female blackflies from releasing the microfilariae.

In July 2015, controlled trials were conducted to learn whether or not adding doxycycline (Acticlate, Doryx, Vibra-Tabs) to the ivermectin would be more effective in treating onchocerciasis. The results were unclear, in part due to issues in how the trials were conducted.

Complications

Nodding disease, which is a rare form of epilepsy, has been associated with onchocerciasis. It's relatively rare, affecting somewhere around 10,000 children in eastern Africa. Trials are being conducted to learn whether or not doxycycline could help reduce the neuroinflammation that occurs.

Public Health Risk

Black flies can be annoying biting pests, but none are known to transmit disease agents to humans in the U. S. However, they transmit one parasitic nematode worm that infects humans in other regions of the world. *Onchocerca volvulus* causes a significant human disease known as onchocerciasis or "river blindness" in equatorial Africa and mountainous regions of northern South America and Central America.

The bites of black flies cause different reactions in humans, ranging from a small puncture wound where the original blood meal was taken to a swelling that can be the size of a golf ball. Reactions to black fly bites that collectively are known as "black fly fever" include headache, nausea, fever, and swollen lymph nodes in the neck.

In eastern North America, only about six black fly species are known to feed on humans. Several other species are attracted to humans, but they typically do not bite. However, the non-biting species fly around the head and may crawl into the ears, eyes, nose, or mouth, causing extreme annoyance to anyone engaged in outdoor activities.

Black flies can be found throughout most of the U. S., but their impact on outdoor activities varies depending on the specific region and time of year. For example, in parts of the upper Midwest and the Northeast, black fly biting can be so extreme, especially in late spring into early summer, it may disrupt or prevent outdoor activities such as hiking, fishing, and kayaking.

Besides being a nuisance to humans, black flies can pose a threat to livestock. They are capable of transmitting a number of different disease agents to livestock, including protozoa and nematode worms, none of which cause disease in humans. In addition to being vectors of disease agents, black flies pose other threats to livestock. For example, when numerous enough, black flies have caused suffocation by crawling into the nose and throat of pastured animals. On rare occasions, black flies have been known to cause exsanguination (death due to blood loss) from extreme rates of biting. Saliva injected by biting black flies can cause a condition known as "toxic shock" in livestock and poultry, which may result in death.

Control

Control of black flies is difficult, typically aimed at the larval stages, and usually involves aerial applications of insecticides or physically altering the habitat of pest species. The most effective control programs are conducted by state agencies or by professional pest control companies contracted by the state. Any effect is limited in duration, however, in large part because females of pest species are capable of flying long distances from the larval developmental site, and they soon re-infest treated areas.

There is little that an affected homeowner or person engaging in outdoor activities can do to control black flies. For personal protection, it is best to avoid peak periods of black fly activity. Information pertaining to the predicted "black fly season" in a particular area often can be obtained by contacting a local Cooperative Extension office. When venturing outdoors in infested areas, apply an insect repellent containing DEET, wear protective clothing, and minimize openings such as buttonholes through which black flies crawl in an attempt to feed. Outdoor activities in heavily infested areas may require the wearing of fine-mesh head nets, similar to those worn by beekeepers.

Management

Control of black flies is difficult because of the number of potential breeding sites. However, satisfactory control has been attained in some states by treating streams with the natural product, *Bacillus thuringiensis* var. *israeliensis*. The breeding sites in streams as well as the potential migration from these sites by the adult female black fly limits the use of chemical pesticides to regulate populations. Breeding site treatments have been used in Africa, but generally have not proven effective. Treatment of breeding sites (streams, rivers, etc.) would involve techniques similar to those used by mosquito control districts for treatment of mosquito larvae in aquatic habitats. Fogging for black flies would have to be done during the day when these insects are actively feeding and when fogging is least effective. These techniques do not seem effective against black flies in the north-eastern United States.

Black flies are small enough to pass through window screen or come indoors on or in the hair. They do, however, prefer to bite out of doors. Long sleeve shirts, long pants and fine screen netting overhead help prevent feeding. Repellents containing "DEET" formulations are not very effective for prevention of black fly bites. Individuals wearing DEET may actually have more black flies attracted to them than individuals not wearing DEET. Our tests indicate that some protection is given by herbal-based treatments with an active ingredient of geraniol. Permethrin products designed specifically to repel ticks also work for black flies as a clothing treatment, but can only be applied to fabrics, such as hats and shirts, and not to skin. Because black flies feed only during the day, our best advice is to limit your exposure to black flies. If this is not possible, try the available repellents in the hope that one of them will be effective for you in protecting against the black flies' bites.

Probable question:

1. How many types of black flies are there?
2. Describe the life cycle of black fly.
3. Describe the larval stage of black fly with diagram.
4. Discuss the biting nature of female black fly.
5. Describe the causative factor, mode of transmission and prophylaxis of river blindness.
6. Write short note on public health risk of black fly.

Suggested readings:

1. Bernays, E.A. and Chapman, R.F. (). Host Selection by Phytophagous insects. Chapman and Hall, New York, USA
2. Gullan, P.J. and Cranston, P.S. (). The Insects: An Outline of Entomology. Wiley Blackwell.
3. Hati, A.K. (2010). Medical Entomology. Allied Book Agency, Kolkata.
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Unit XII
Life cycle, mode of transmission, control and importance of Flea

Objective: In this unit you will know about life cycle, mode of transmission, control and importance of Flea.

Introduction

Flea, (order Siphonaptera), any of a group of bloodsucking insects that are important carriers of disease and can be serious pests. Fleas are parasites that live on the exterior of the host (i.e., are ectoparasitic). As the chief agent transmitting the Black Death (bubonic plague) in the Middle Ages, they were an essential link in the chain of events that resulted in the death of a quarter of the population of Europe.

Systematic Position:

Kingdum: Animalia

Phylum: Arthropoda

Class : Insecta

Order : Siphonaptera

Family : Pulicidae

Genus : *Xenopsylla*

Species: *cheopsis* (Rothschild, 1903)

Habits of Flea

Distribution: Flea are found throughout the world on their normal host, in the nest, burrow and hair of their host.

Bites: Both the sexes bite and suck the blood from their host.

Feeding: Both the sexes are ecto-parasites and feed usually once a day.

Dispersal: Flea cannot fly but they jump about 4 inches when starved. Mainly flea are disperse by their host.

Life period: Adult flea may live up to one year.

General Features

Fleas are small, wingless insects with a tough cuticle bearing many bristles and frequently combs (ctenidia) of broad, flattened spines (Fig 1). The adult flea varies from about 0.1 to 1 cm (0.039 to 0.39 inch) in length and feeds exclusively on the blood of mammals (including humans) and birds. With about 2,000 species and subspecies known, the order is still a small one compared with many other groups of insects. However, it is widely distributed with some—such as the rat flea and the mouse flea—having been carried all over the world by humans. Native species of fleas are found in polar, temperate, and tropical regions. e.g., Oriental rat flea (*Xenopsylla cheopis*).

The Oriental rat flea (*Xenopsylla cheopis*), also known as the tropical rat flea, is a parasite of rodents, primarily of the genus *Rattus*, and is a primary vector for bubonic plague and murine typhus. This occurs when the flea has fed on an infected rodent, and then bites a human.

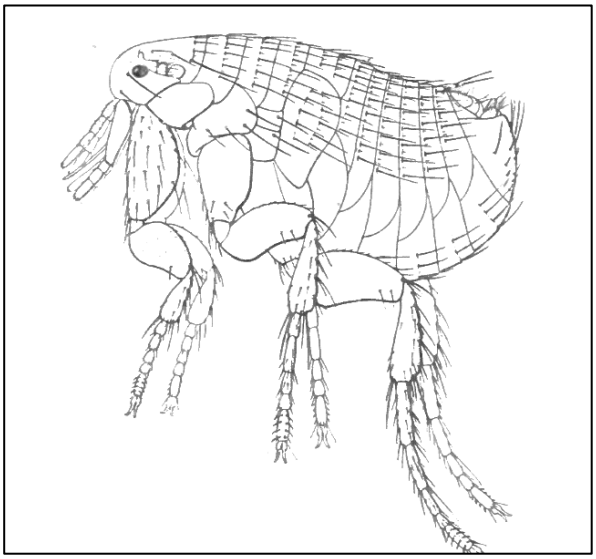

Fig 1: Flea (*Xenopsylla cheopis*)

1. It is commonly known as (rat flea or Pissu), and is common in tropics.

2. It is an ectoparasite on rats and other such mammals and acts as disease vector for plague.

3. Body is laterally compressed and maybe divided into head thorax and abdomen, which may not be demarcated clearly.

4. The head bears a small 3 to 4 jointed antennae a pair of simple eyes and piercing & sucking or siphoning mouthparts.

5. Thorax bears three pairs of long, jointed and clawed legs for hopping but no wings.

6. Abdomen is swollen in middle and has 8 segments and a pair of anal styles.

7. The whole body is covered over with bristles on the dorsal as well as lateral sides.

Life cycle:

Details of the life cycle are available for only a few species. The four life stages are the egg, larva, pupa, and adult (Fig 2).

Eggs: Pearly white, oval, microscopic eggs fall easily from the female to the ground or from the animal she lays on. If they are laid on an animal, they soon fall off in the dust or in the animals bedding. If the eggs do fall immediately on the ground, then they fall into crevices on the floor where they will be safe until they hatch one to ten days later; when environmental conditions are just right for them. If temperatures are cold and dry, the eggs will take longer; if temperatures are warm and humidity levels are high, the eggs will hatch at a faster rate. Larvae then emerges as the next life stage

Larva: Larva looks very similar to a worm and is about 2mm long and legless. It only has a small body and a mouth part. At this stage, the flea does not drink blood; instead it feeds on organic debris, such as dried excrement, dried bits of skin, dead mites, dried blood found in the host's nest and other smaller parasites lying around them in the dust.

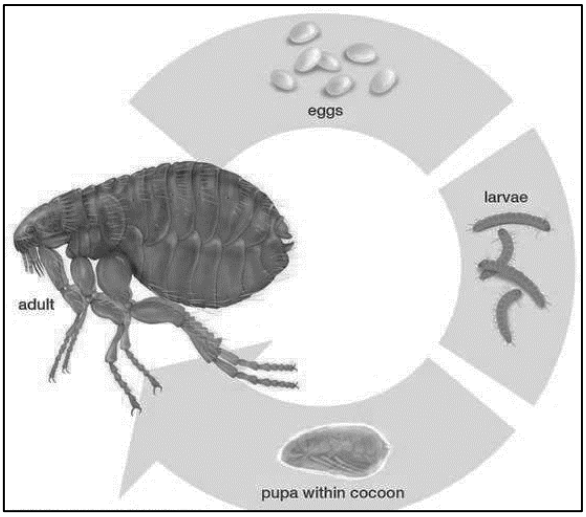

Fig 2: Stages in the life cycle of fleas.

Pupa: After three (exceptionally, two) moults, the larva spins a silk cocoon that includes debris from the nest and enters the pupal stage. Cocoons have a sticky outer coating that allows them to hide deep in the carpeting and not be easily removed by light vacuuming or sweeping. The cocoon also serves to protect the developing adults from chemicals. The flea remains a pupa from one week to six months changing in a process called metamorphosis. The adult fleas pass freshly imbibed blood rapidly through their gut to produce faecal matter for the nourishment of their offspring, which is essential to the successful metamorphosis of certain species of flea larvae.

Adult: Some species can enter an arrested state of development at the end of the pupal stage and will not emerge as an adult until a host is present. Depending upon the species or environmental conditions, the time required for a complete life cycle varies from two weeks to several months. The life span of the adult flea varies from a few weeks (e.g., *Echidnophaga gallinacea*) to a year or more (*Pulex irritans*). The life cycle of the European rabbit flea (*Spilopsyllus cuniculi*) and its host are perfectly synchronized. When the flea emerges, it begins the final cycle, called the adult stage.

It will need to begin feeding from a host within a few hours. Shortly after the first meal, adult fleas will breed and begin laying eggs within a few days. Female fleas are not able to lay eggs (up to 50 eggs per day) until they obtain a blood meal.

New adult fleas have a flat-bodied appearance and are very small and dark in color. Once they have had a chance to feed off your pet, they will become larger and lighter in color, taking on the more recognizable flea shape. Adult fleas account for less than 5% of the entire flea population in a home. They spend the majority of their time living on the host while they feed, breed, and lay eggs, and can live anywhere from a couple of weeks to several months on the host animal.

Form and Function

Anatomically, adult fleas are a rather uniform but distinctive group, with many interesting modifications and few obvious links with other orders. The compressed body enables them to move swiftly through hairs or feathers of the host, while the backwardly projecting spines or combs serve to anchor them within fur, hair, or feathers. The mouthparts are modified for sucking blood and include barbed stylets that aid both in penetration of the flea into the host skin and in attachment of species that spend long periods fixed to the host (e.g., the stick tight fleas). Generally, fleas that live on diurnal hosts have well-developed eyes, whereas species that parasitize subterranean hosts (e.g., moles) or nocturnal animals (e.g., bats) have poorly developed eyes or none at all (Fig 3).

The most impressive adaptations are highly developed jumping legs. During their evolution, fleas, like the majority of parasitic insects, lost their wings. However, certain parts of the flight mechanism have been retained and incorporated in the jumping mechanism. For example, on flying insects, a rubbery protein known as resilin forms a hinge where the wings attach to the body.

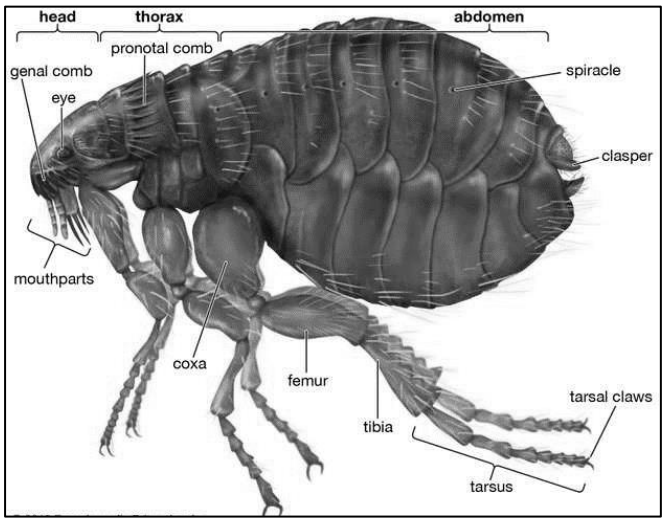

Fig 3: Eternal morphology of adult flea

Resilin absorbs compression and tension created during each wing stroke, and the stored energy is transferred through an elastic recoil-like effect to assist in the initiation of each successive stroke. Fleas, despite their wingless state, have retained the resilin hinge on the thorax at the site where the legs attach to the body. When a flea crouches, the resilin pads become compressed, and they are maintained in this state by a muscle-controlled catch mechanism. In the instant prior to jumping, the catch muscles relax, and the energy in the resilin pads is transferred through the legs. This creates a lever effect that pushes each tibia and tarsus onto the ground and thereby causes the flea to jump.

Because fleas are able to leap horizontal or vertical distances 200 times their body length and to develop an acceleration of 200 gravities, they have been described as insects that fly with their legs. Certain species that live in nests high above the ground or in other unusual habitats crawl rather than jump. An incidental use of the unusual strength of fleas is in "flea circuses" in which they pull miniature carts and perform other feats.

1. Oriental rat flea (*Xenopsylla cheopis*)

Structure

The Oriental rat flea has no genial or pronotal combs. This characteristic can be used to differentiate the oriental rat flea from the cat flea, dog flea, and other fleas.

The flea's body is only about one tenth of an inch long (about 2.5 mm). A flea's body is constructed to make it easier to jump long distances. The flea's body consists of three regions: head, thorax, and abdomen. The head and the thorax have rows of bristles (called combs), and the abdomen consists of eight visible segments.

A flea's mouth has two functions: one for squirting saliva or partly digested blood into the bite, and one for sucking up blood from the host. This process mechanically transmits pathogens that may

cause diseases the flea might have. Fleas smell exhaled carbon dioxide from humans and animals and jump rapidly to the source to feed on the newly found host. A flea is wingless so it cannot fly, but it can jump long distances with the help of small, powerful legs. A flea's leg consists of four parts: the part that is closest to the body is the coxa; next are the femur, tibia, and tarsus. A flea can use its legs to jump up to 200 times its own body length (about 20 in or 50 cm).

Life cycle

Fleas, like other holometabolous insects, have a four-part life cycle consisting of eggs, larvae, pupae, and adults (Fig 4). Eggs are shed by the female in the environment Eggs hatch into larvae in about 3-4 days and feed on organic debris in the environment. The number of larval instars varies among the species. Larvae eventually form pupae, which are in cocoons that are often covered with debris from the environment (sand, pebbles, etc). The larval and pupal stages take about 3-4 weeks to complete. Afterwards, adults hatch from pupae and seek out a warm-blooded host for blood meals. The primary hosts for *Ctenocephalides felis* and *C. canis* are cats and dogs, respectively, although other mammals, including humans, may be fed upon. The primary hosts for *Xenopsylla cheopis* are rodents, especially rats. In North America, plague (*Yersinia pestis*) is cycled between *X. cheopis* and prairie dogs. Humans are the primary host for *Pulex irritans.*

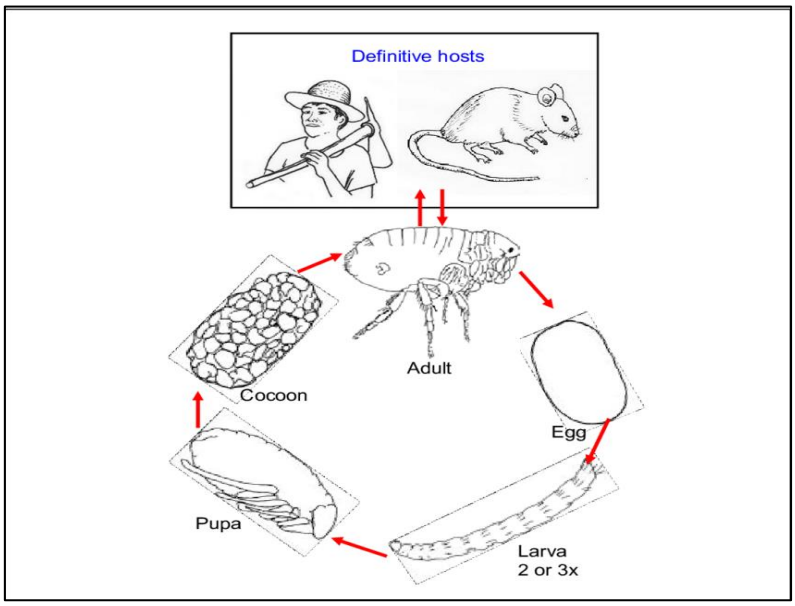

Fig 4: Life cycle of *Xenopsylla cheopis*

I. Bubonic Plague:

The three types of plague are the result of the route of infection: bubonic plague, septicemic plague, and pneumonic plague.

Plague causes more human deaths than any other infectious diseases except malaria and tuberculosis. It is caused by *Yersinia pestis*, an aerobic gram-negative rod-shaped bacterium. Plague is a natural disease of domestic and wild rodents; rats being the primary disease reservoir, Fleas are the intermediate hosts responsible to spread the disease from rats to mammals (Fig 4). Rat flea (*Xenopsylla cheopis*) ingests the pathogen by sucking blood from an infected rat. Bacteria multiply in flea's intestine and can be transmitted to a healthy animal (including man) in the next bite. Once in man's body, the pathogens reach the lymph nodes where they result in swollen areas called buboes

hence the disease called bubonic plague. Three to seven days after exposure to the bacteria flu like symptoms develop. This includes fever, headaches, and vomiting.

If not treated in the early stage, the disease usually causes death within 3-5 days. When *Y. pestis* cells are either inhaled directly or reach the lungs during bubonic plague, they cause pneumonic plague. Symptoms are usually absent until the last day or two when large amounts of bloody sputum are produced. Untreated victims rarely survive more than two days.

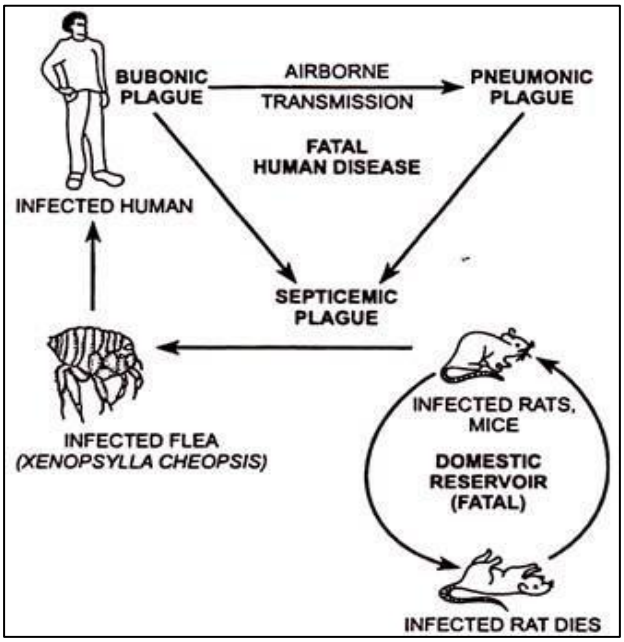

Fig 4: Plague pathogenesis

Septisemic plague is caused due to the rapid spread of *Y. pestis* throughout the human body via the bloodstream without the development of bulboes and usually results in death before the diagnosis could be made. *Y. pestis* bacilli can resist phagocytosis and even reproduce inside phagocytes and kill them. As the disease progresses, the lymph nodes can hemorrhage and become swollen and necrotic. Bubonic plague can progress to lethal septicemic plague in some cases. In very rare circumstances, as in the septicemic plague, the disease can be transmitted by direct contact with infected tissue or exposure to the cough of another human. The flea is parasitic on house and field rats, and seeks out other prey when its rodent hosts die. The bacteria remained harmless to the flea, allowing the new host to spread the bacteria. The bacteria form aggregates in the gut of infected fleas and this results in the flea regurgitating ingested blood, which is now infected, into the bite site of a rodent or human host. Once established, bacteria rapidly spread to the lymph nodes and multiply.

The plague is also known to spread to the lungs and become the disease known as the pneumonic plague.

Flea bites. Plague bacteria are most often transmitted by the bite of an infected flea. During plague epizootics, many rodents die, causing hungry fleas to seek other sources of blood. People and animals that visit places where rodents have recently died from plague are at risk of being infected from flea bites. Dogs and cats may also bring plague-infected fleas into the home. Flea bite exposure may result in primary bubonic plague or septicemic plague.

Fleas become infected by feeding on rodents, such as the chipmunks, prairie dogs, ground squirrels, mice, and other mammals that are infected with the bacteria Yersinia pestis. Fleas transmit the plague

bacteria to humans and other mammals during the feeding process. The plague bacteria are maintained in the blood systems of rodents.

Mode of transmission

This species can act as a vector for plague, *Yersinia pestis*, *Rickettsia typhi* and also act as a host for tapeworms *Hymenolepis diminuta* and *Hymenolepis nana*. Diseases can be transmitted from one generation of fleas to the next through the eggs. *Xenopsylla cheopis* was undoubtedly responsible for the spread of the "Black death" in Ireland in the 14th Century.

Biting: The chief method of transmission in case of plague is the bite of hungry blocked flea. Some flea which ingest plague bacilli become blocked due to the multiplication of plague bacilli in their stomach. Flea affected in this way are called blocked flea, so due to this blockage flea are unable to obtain further blood feed. Because of hunger, flea begin to bite more forcefully to suck the blood, so instead of sucking blood it injects plague bacilli to the wound. Such blocked flea play a great role in the spread of plague.

Mechanical transmission: It takes place from the proboscis of the flea, which had fed recently on infected rodent.

Faeces: The faecal drop of infected flea may contain numerous bacilli. When the host scratches the flea bitten area, there is a direct inoculation of the infected agents in to the injury spot.

II. Murine Typhus:

Murine typhus (also called endemic typhus) is a form of typhus transmitted by fleas (*Xenopsylla cheopis*), usually on rats. (This is in contrast to epidemic typhus, which is usually transmitted by lice.) Murine typhus is an under-recognized entity, as it is often confused with viral illnesses. Most people who are infected do not realize that they have been bitten by fleas.
It is caused by the bacteria *Rickettsia typhi*, and is transmitted by the fleas that infest rats. While rat fleas are the most common vectors, cat fleas and mouse fleas are less common modes of transmission. These fleas are not affected by the infection. Human infection occurs because of flea-fecal contamination of the bites on human skin. Rats, cats, possums maintain the rickettsia colonization by providing it with a host for its entire life cycle. Rats can develop the infection, and help spread the infection to other fleas that infect them, and help multiply the number of infected fleas that can then infect humans.

Less often, endemic typhus is caused by *Rickettsia felis* and transmitted by fleas carried by cats or opossums.

- Primarily via infected flea feces scratched into skin or rubbed into an eye.
- NOTE: Transovariole transmission has been documented in female Oriental rat fleas.
- NOTE: Laboratory research suggests that transmission is possible via bites of the cat flea.

III. Cat Scratch Disease (Bartonellosis)

Cat scratch disease is a bacterial disease caused by *Bartonella henselae*. People with weak immune systems are at increased risk of getting seriously ill with cat scratch fever. Young cats and kittens are most likely to be the source of human infection and about 40% of cats carry these bacteria at some point in their lives. The infection, which rarely causes disease in cats, is transmitted between cats by fleas. Infected flea droppings on the cat's fur or claws are the source of human infections, which are

spread from the cat to a person by a cat bite, scratch or lick. Cat scratch fever can be prevented by practicing effective flea control and by avoiding cat bites or scratches.

IV. Tungiasis

A tropical ailment caused by the chigoe flea when it burrows into the skin and takes a blood meal. The bite of the chigoe flea often results in secondary infections and itching.

Importance

Infestation by fleas may cause severe inflammation of the skin and intense itching. Although many animals acquire partial immunity after constant or repeated attacks, individuals (especially humans) can occasionally become sensitized after exposure and develop allergies.

Species that attack people and livestock include the cat flea (*Ctenocephalides felis*), the so-called human flea (*Pulex irritans*), the dog flea (*Ctenocephalides canis*), the stick tight flea (*Echidnophaga gallinacea*), and the jigger, or chigoe, flea (*Tunga penetrans*). Poultry may be parasitized by the European chicken flea (*Ceratophyllus gallinae*) and, in the United States, by the western chicken flea (*Ceratophyllus niger*). Certain fleas that feed primarily on rodents or birds sometimes attack people, particularly in the absence of their usual host.

When rats are dying of bubonic plague, their hungry fleas, themselves infected with plague bacilli and seeking food elsewhere, can transmit the disease to humans, especially in buildings heavily infested with rats. The Oriental rat flea (*Xenopsylla cheopis*) is the most efficient carrier of plague, but other species of fleas (e.g., *Nosopsyllus fasciatus*, *Xenopsylla brasiliensis*, *Pulex irritans*) can also transmit the disease to people. Although there are occasional cases of plague in tropical and some temperate regions, the disease in humans can be controlled by early diagnosis and antibiotics. Plague (sylvatic plague) is a widespread disease in hundreds of species of wild rodents throughout the world and is maintained in those populations by fleas that parasitize these animals. More than 100 species of fleas are known to be able to be infected by the plague bacillus, and an additional 10 species are implicated as carriers of the classic type of urban plague.

Fleas, particularly *Xenopsylla cheopis*, are thought to be the principal carriers of murine (endemic) typhus, a rickettsial disease of humans. As in plague, rats and mice are the sources of infection. Fleas are considered important in the maintenance and spread of many locally restricted infections among rodents and other mammals, including tularemia and Russian spring-summer encephalitis.

Fleas transmit myxomatosis, a viral disease of rabbits, which is used deliberately to control rabbits in areas where they are severe pests (e.g., in Australia). Fleas are probable carriers of a filarial worm of dogs and serve as the intermediate host of a common tapeworm (*Dipylidium caninum*) of dogs and cats and occasionally children. If heavily infested, animals can suffer severe damage or be killed by the effects of flea bites and the resultant loss of blood.

Fleas are subject to parasitism by external mites, internal nematode worms, and bacterial, fungal, and protozoan infections. The female jigger, or chigoe, flea (not to be confused with “chigger,” a larval mite) burrows into the skin of its host, generally on the feet, and lives within a cyst that forms around it. Intense itching accompanies the development and enlargement of the cyst as the abdomen of the pregnant flea swells to the size of a pea; secondary infections may constitute serious complications.

Control

In controlling fleas it is best to treat simultaneously both the host nest and bedding area, which is the breeding site of fleas, and the infested host, The larval and pupal stages usually develop away from the host's body.

For infested animals a commercial dust, spray, dip, or aerosol containing an insecticide or growth regulator is used. However, in some regions, fleas have become resistant to some insecticides, and new materials are required.

For the control of larval and adult fleas away from the host, insecticides or growth regulators may be applied to the pens and haunts of the affected animals.

Repellents may be effective in preventing attack by fleas.

Probable questions:

1. Write down the systematic position of flea.
2. Describe the morphology of *Xenopsylla cheopis*.
3. Discuss the life cycle of flea.
4. Describe the pathogenicity, prophylaxis of bubonic plague.
5. Describe the pathogenicity, prophylaxis of murine typhus.

Suggested readings:

1. Bernays, E.A. and Chapman, R.F. (). Host Selection by Phytophagous insects. Chapman and Hall, New York, USA
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Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-IV

HARD CORE THEORY PAPER

MOLECULAR BIOLOGY &

BIOTECHNOLOGY & TOOLS &TECHNIQUE

ZHT-412

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

W.B. INDIA

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Director
Prof. Manas Mohan Adhikary
Directorate of Open and Distance Learning
University of Kalyani

HARD CORE THEORY PAPER (ZHT -412)

MOLECULAR BIOLOGY AND BIOTECHNOLOGY AND TOOLS AND TECHNIQUE

Group A: MOLECULAR BIOLOGY AND BIOTECHNOLOGY

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZHT - 412 (MOLECULAR BIOLOGY AND BIOTECHNOLOGY AND TOOLS AND TECHNIQUE)	I	Transcriptional control of gene expression- positive and negative regulations, RNA polymerases, promoters and regulatory sequences, activators and repressors of transcription, transcription initiation by RNA polymerases, regulation of transcription factor activity, elongation and termination of transcription.	1.5	1	1	
	II	Post-transcriptional gene control -types of introns and their splicing, evolution of introns, catalytic RNA, alternative splicing and proteome diversity, regulation of Pre-mRNA Processing, micro RNA and other non-coding RNAs, degradation of RNA.		1	1	
	III	Transport across the nuclear envelope and stability of RNA-structure of nuclear membrane and nuclear pore complexes, processes of nuclear import and export and their regulation, degradation of RNA. Translational machinery and translational control - energetics of amino acid polymerization, tRNAs and their modifications, aminoacyl tRNA synthetases, accuracy during aminoacylation		1	1	

		of tRNA, regulation of initiation of translation in eukaryotes, elongation and its control, inhibitors of translations.				
	IV	**Basic recombinant DNA techniques, cutting and joining DNA molecules, restriction modification systems, various enzymes used in recombinant DNA technology, restriction maps and mapping techniques; footprinting, methyl interference assay. Polymerase chain reaction– methods and applications.**		**1**	**1**	
	V	**Basic biology of cloning vectors: plasmids, phages, single stranded DNA vectors, high capacity vectors, retroviral vectors, expression vectors and other advanced vectors in use. Gene cloning strategies: methods of transforming E. coli with rDNA; methods of selection and screening of transformed cells; construction of genomic and cDNA libraries; phage display.**		**1**	**1**	
	VI	**Manipulating genes in animals: gene transfer to animal cells, genetic manipulation of animals, transgenic technology, application of recombinant DNA technology; genetically modified organisms: gene knockouts, mouse disease models, gene silencing, gene therapy, somatic and germ- line therapy**		**1**	**1**	

Unit-I

Transcriptional control of gene expression- positive and negative regulations, RNA polymerases, promoters and regulatory sequences, activators and repressors of transcription, transcription initiation by RNA polymerases, regulation of transcription factor activity, elongation and termination of transcription

Objective: In this unit you will know about various aspects of transcription i.e. initiation, elongation and termination. You will also know about Gene regulation mechanisms in prokaryotes and eukaryotes. There will also be discussion about transcription regulatory events.

Introduction:

Transcription is a process in which ribonucleic acid (RNA) is synthesized from DNA. The word gene refers to the functional unit of the DNA that can be transcribed. Thus, the genetic information stored in DNA is expressed through RNA. For this purpose, one of the two strands of DNA serves as a template (non-coding strand or sense strand) and produces working copies of RNA molecules.

The other DNA strand which does not participate in transcription is referred to as coding strand or antisense strand (frequently referred to as coding strand since with the exception of T for U, primary mRNA contains codons with the same base sequence).

Transcription is Selective:

The entire molecule of DNA is not expressed in transcription. RNAs are synthesized only for some selected regions of DNA. For certain other regions of DNA, there may not be any transcription at all. The exact reason for the selective transcription is not known. This may be due to some inbuilt signals in the DNA molecule.

The product formed in transcription is referred to as primary transcript. Most often, the primary RNA transcripts are inactive. They undergo certain alterations (splicing, terminal additions, base modifications etc.) commonly known as post- transcriptional modifications, to produce functionally active RNA molecules. There exist certain differences in the transcription between prokaryotes and eukaryotes. The RNA synthesis in prokaryotes is given in some detail. This is followed by a brief discussion on eukaryotic transcription.

Transcription in Prokaryotes:

A single enzyme—DNA dependent RNA polymerase or simply RNA polymerase—synthesizes all the RNAs in prokaryotes. RNA polymerase of E. coli is a complex holoenzyme (mol wt. 465 kDa) with five polypeptide subunits— 2α, 1β and 1β' and one sigma (σ) factor (Fig. 4.2). The enzyme without sigma factor is referred to as core enzyme ($\alpha_2\beta\beta'$).

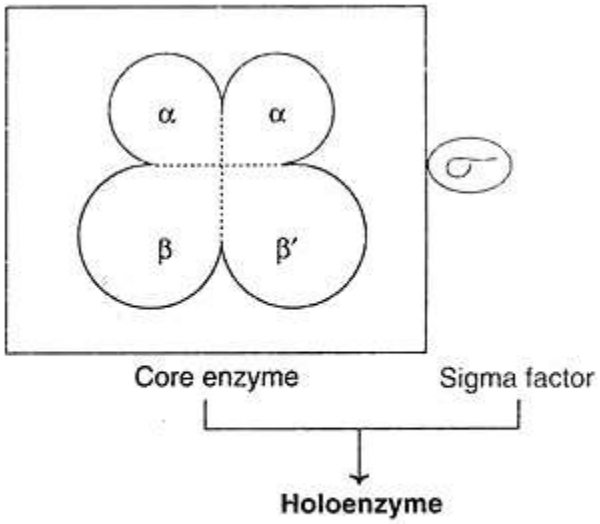


Fig. 4.2 : RNA polymerase of E. coli.

Initiation:

The binding of the enzyme RNA polymerase to DNA is the prerequisite for the transcription to start. The specific region on the DNA where the enzyme binds is known as promoter region. There are two base sequences on the coding DMA strand which the sigma factor of RNA polymerase can recognize for initiation of transcription (Fig. 4.5).

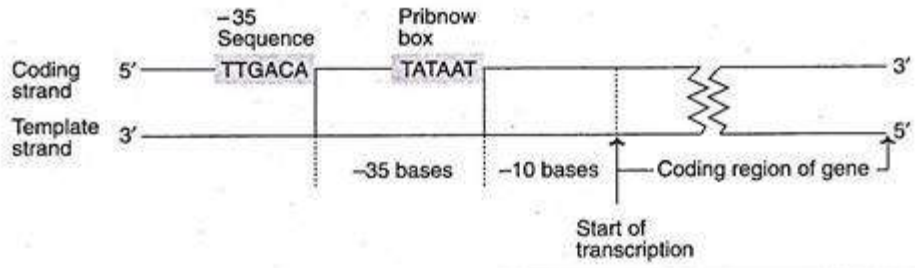


Fig. 4.5 : Promoter regions of DNA in prokaryotes.

1. Pribnow box (TATA box):

This consists of 6 nucleotide bases (TATAAT), located on the left side about 10 bases away (upstream) from the starting point of transcription.

2. The '-35' sequence:

This is the second recognition site in the promoter region of DNA. It contains a base sequence TTGACA, which is located about 35 bases (upstream, hence -35) away on the left side from the site of transcription start.

The initiation consists of the following steps:

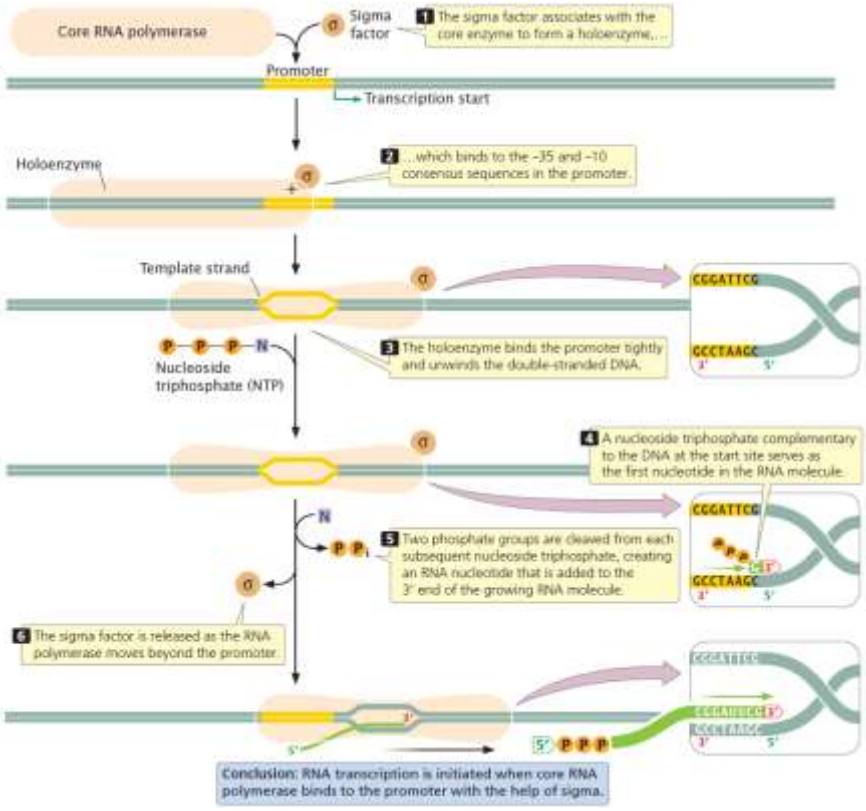
(i) RNA polymerase (RNAP) binds to one of several specificity factors, to form a holoenzyme. In this form, it can recognize and bind to' specific promoter regions in the DNA. At this stage,

the DNA is double-stranded ("closed"). This holoenzyme/wound-DNA structure is referred to as the closed complex.

(ii) The DNA is unwound and becomes single-stranded ("open") in the vicinity of the initiation site (defined as + 1). This holoenzyme/unwound-DNA structure is called the open complex.

(iii) The RNA polymerase transcribes the DNA, but produces about 10 abortive (short, non-productive) transcripts which are unable to leave the RNA polymerase because the exit channel is blocked by the cr-factor.

(iv) The a-factor eventually dissociates from the holoenzyme, and elongation proceeds. Most transcripts originate using adenosine-5′-triphosphate (ATP) and, to a lesser extent, guanosine-5′-triphosphate (GTP) (purine nucleoside triphosphates) at the +1 site. Uridine-5′- triphosphate (UTP) and cytidine-5′-triphosphate (CTP) (pyrimidine nucleoside triphosphates) are disfavoured at the initiation site.



2. Elongation:

In transcription only one strand of DNA [called template strand or non-coding strand] takes part as a template. As transcription proceeds, RNA polymerase traverses the template strand and uses base pairing complementarity with the DNA template to create an RNA copy.

Although RNA polymerase traverses the template strand from 3′ —> 5′, the coding (non-template) strand is usually used as the reference point, so transcription is said to go from 5′ -> 3′.This produces an RNA molecule from 5′ 3′, an exact copy of the coding strand (except that thymine are replaced with uracil, and the nucleotides are composed of a ribose (5-carbon) sugar where DNA has deoxyribose (one less oxygen atom) in its sugar-phosphate backbone).

In the prokaryotes, the elongation starts with the **"abortive initiation cycle".** During this cycle RNA Polymerase will synthesize mRNA fragments 2-12 nucleotides long. This continues to occur until the σ factor rearranges, which results in the transcription elongation complex (which gives a 35 bp moving footprint). The a factor is released before 80 nucleotides of mRNA are synthesized.

3. Termination:

In prokaryotes, two different modes of transcription termination, viz:

(i) Rho-independent and
(ii) Rho-dependent are well known.
These are briefly discussed as follows:

(i) Rho-independent termination:
It is also known as intrinsic transcription termination. It involves terminator sequences within the RNA that signal the RNA polymerase to stop. The terminator sequence is usually A palindromic sequence that forms a stem-loop hairpin structure that leads to the dissociation of the RNAP from the DNA template.
In the Rho-independent transcription termination, RNA transcription stops when the newly synthesized RNA molecule forms a G-C rich hairpin loop, followed by a run of U's, which makes it detached the DNA template.

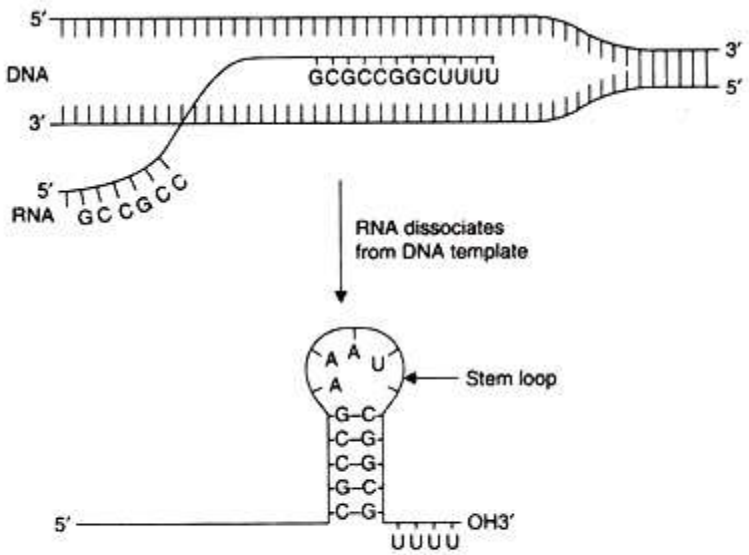


Fig. 15.8 Termination of transcription is signaled by a GC-rich inverted repeat followed by four A residues. The formation of a stem-loop structure by the inverted repeat in the RNA, causes RNA to dissociate from the DNA template.

(ii) Rho-dependent termination:

In the **"Rho-dependent"** type of termination, a protein factor called **"Rho"** is used to stop RNA synthesis at specific sites. This protein binds at a Rho utilisation site on the nascent RNA strand and runs along the mRNA towards the RNA polymerase.

When p-factor reaches the RNAP, it causes RNAP to dissociate from the DNA, terminating transcription. In other words, it destabilizes the interaction between the template and the mRNA, thus releasing the newly synthesized mRNA from the elongation complex.

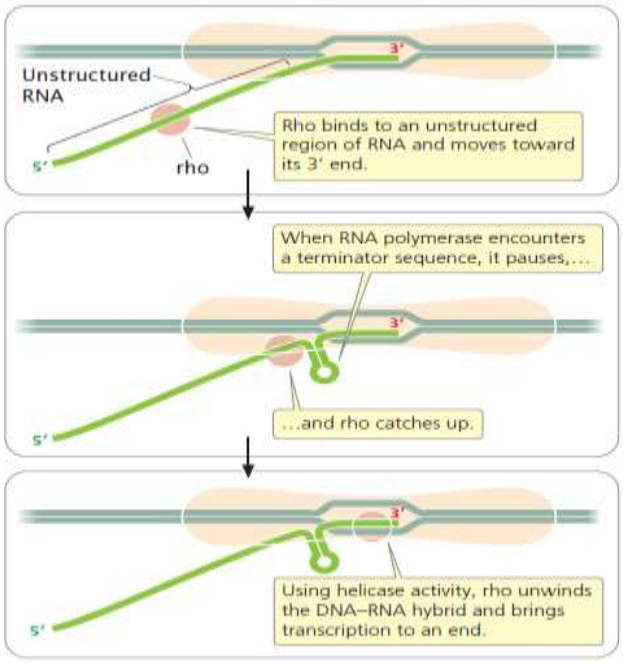


Figure: Rho-dependent terminator

Reverse Transcription in Prokaryotes:

Synthesis of DNA from RNA molecule in the presence of enzyme reverse transcriptase is referred to as reverse transcription. Reverse transcription was first reported by Temin and Baltimore in 1970 for which they were awarded Nobel Prize in 1975.

Reverse transcription is also known as Teminism. Some viruses (such as HIV, the cause of AIDS), have the ability to transcribe RNA into DNA. HIV has an RNA genome that is duplicated into DNA. The resulting DNA can be merged with the DNA genome of the host cell. The main enzyme responsible for synthesis of DNA from an RNA template is called reverse transcriptase. In the case of HIV, reverse transcriptase is responsible for synthesizing a complementary DNA strand (cDNA) to the viral RNA genome.

An associated enzyme, ribonuclease H, digests the RNA strand, and reverse transcriptase synthesizes a complementary strand of DNA to form a double helix DNA structure. This cDNA is integrated into the host cell's genome via another enzyme (integrase), causing the host cell to generate viral proteins which reassemble into new viral particles. Subsequently, the host cell undergoes programmed cell death (apoptosis).

TABLE 21.1. Differences between transcription and reverse transcription

S.No.	*Particulars*	*Transcription*	*Reverse Transcription*
1.	Occurs in	Both prokaryotes and eukaryotes	Reported in some viruses
2.	Molecule synthesized	RNA from DNA	DNA from RNA
3.	Enzyme involved	Transcriptase	Reverse transcriptase
4.	Template used	DNA	RNA

Detection of Transcription in Prokaryotes:
Transcription can be measured and detected in a variety of ways.

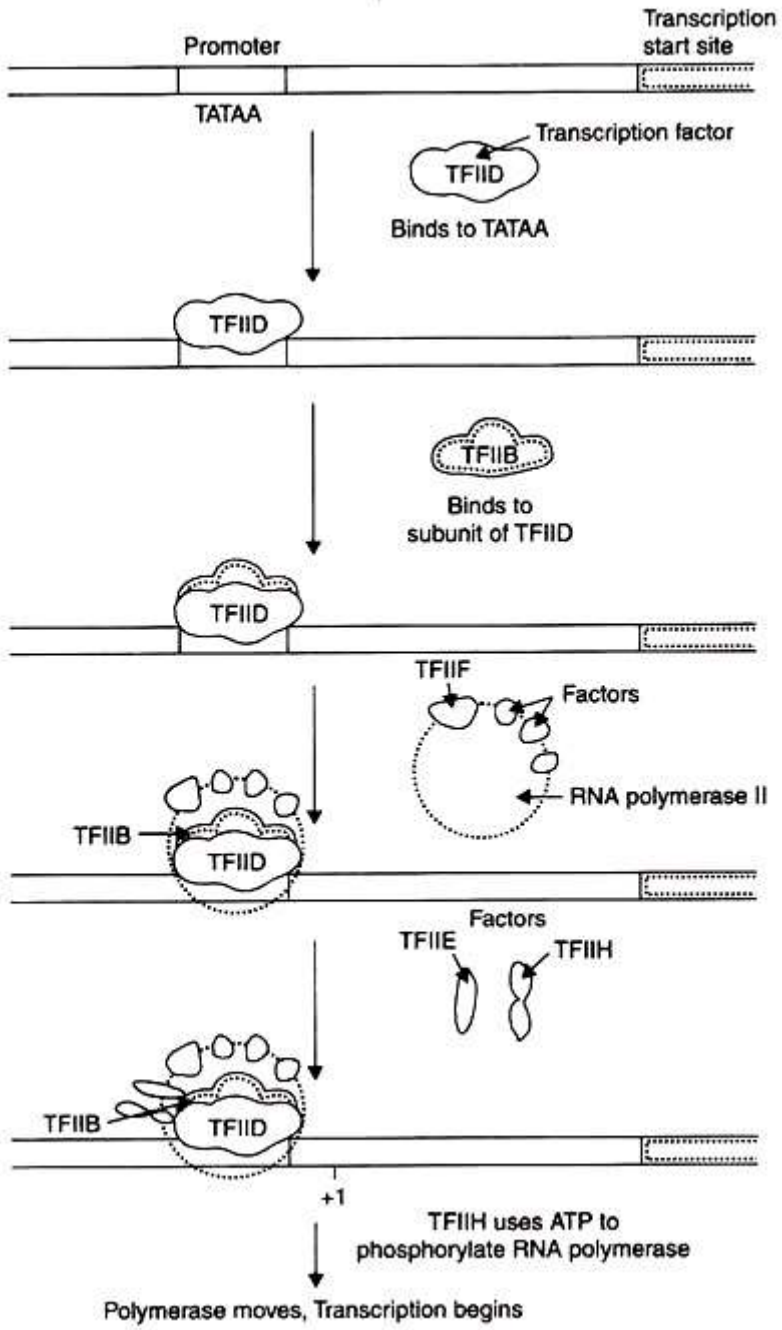
The commonly used methods of detecting transcription are given below:
1. Nuclear Run-on assay, measures the relative abundance of newly formed transcripts.
2. RNAse protection assay and ChlP-Chip of RNAP, detect active transcription sites.
3. RT-PCR, measures the absolute abundance of total or nuclear RNA levels, which may however-differ from transcription rates.
4. DNA microarrays measures the relative abundance of the global total or nuclear RNA levels, which may however differ from transcription rates.
5. In situ hybridization, detects the presence of a transcript.

Transcription in Eukaryotes:
There are two major differences between prokaryotic and eukaryotic transcription systems. First, a single RNA polymerase is able to transcribe all genes in bacteria, whereas eukaryotic cells have multiple different RNA polymerases that transcribe distinct classes of genes. Second, eukaryotic RNA polymerases do not bind directly to promoter sequences, but interact with a number of proteins to specifically initiate transcription. The complexity of the transcription process in eukaryotes is presumed to be related with the regulation of gene expression required to control activities of many different cell types in multicellular forms. Three distinct nuclear RNA polymerases transcribe different classes of genes in eukaryotic cells (Table).

Table. Classes of genes transcribed by different eukaryotic RNA polymerases

RNA Polymerase	*Type of RNA Synthesised*
II	*m*RNA, small nuclear snRNA
III	*t*RNA, 5S *r*RNA
I	*r*RNA 5.8*S*, 18*S*, 28*S*, small cytoplasmic scRNA

RNA polymerase II transcribes protein coding genes in nucleus to yield mRNAs; RNA polymerases I and III transcribe ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). The three largest species of tRNAs are transcribed by RNA polymerase I. The genes for transfer RNA and the smallest species of ribosomal RNA (5S rRNA), as well as some small nuclear (snRNAs) and cytoplasmic RNAs (scRNAs) involved in splicing and protein transport are transcribed by RNA polymerase III. In addition, mitochondria and chloroplasts contain separate RNA polymerases similar to bacterial RNA polymerases that specifically transcribe DNA in these organelles.



Stages in initiation of transcription

Transcription by RNA Polymerase II:

The different mode of action of transcription in eukaryotic cells was noted in 1979 when it was found that RNA polymerase II is able to initiate transcription only if additional proteins are added to the reaction. In contrast with the bacterial sigma factors, transcription in eukaryotic cells requires distinct initiation factors that were not associated with the polymerase.

Specific proteins acting as transcription factors have now been identified that are required by RNA polymerase II to initiate transcription. Two types of transcription factors have been defined: general transcription factors involved in transcription from all polymerase II promoters; additional transcription factors involved in control of expression of individual genes. Experiments using in vitro systems have indicated that five general transcription factors are required for initiation of transcription by RNA polymerase II. The promoters of many genes

transcribed by polymerase II contain a sequence similar to TATAA 25 to 30 nucleotides upstream of the transcription start site.

This sequence referred to as the TATA box is similar to the -10 sequence of bacterial promoters and is involved in initiation of transcription as follows: first, a general transcription factor called TFIID (TF indicates transcription factor, II denotes polymerase II) binds to the TATA box. TFIID has multiple subunits including the TATA-binding protein (TBP). The TBP binds specifically to the TATAA consensus sequence and 10-12 other polypeptides called TBP-associated factors (TAFs). Second, TBP binds to a second general transcription factor (TFIIB) forming a TBP-TFIIB complex at the promoter. Following recruitment of RNA polymerase II to the promoter, two additional factors (TFIIE and TFIIH) are required for initiation of transcription.

Two subunits of TFIIH are helicase that unwind DNA around initiation site, while another subunit is a protein kinase that phosphorylates repeated sequences in the largest subunit of RNA polymerase II. In spite of the development of in vitro systems, much remains to be elucidated about polymerase II transcription in eukaryotic cells.

Transcription by RNA Polymerases I and III:

Like RNA polymerase II, the other two polymerases I and III also require additional transcription factors to associate with appropriate promoter sequences. Although the three eukaryotic polymerases recognise distinct types of promoters, a common transcription factor, the TATA- binding protein (TBP) seems to be required for initiation of transcription by all 3 polymerases.

RNA polymerase I transcribes ribosomal RNA genes which are present in tandem repeats, to yield a large 45S pre-rRNA, which is then processed to derive the 28S, 18S and 5.8S rRNAs (Fig. 15.9). The promoter of rRNA genes consists of 150 base pairs just upstream of the transcription initiation site. These promoter sequences are recognised by two transcription factors, UBF (upstream binding factor) and SL1 (selectivity factor 1) which bind to the promoter and then recruit polymerase I to form an initiation complex.

One of the four protein subunits of the SL1 transcription factor is TBP. Thus, TBP is a common transcription factor required by all 3 types of eukaryotic RNA polymerases. The promoter of ribosomal RNA genes does not contain TATA box, therefore, TBP does not bind to specific promoter sequences. Thus TBP associates with ribosomal RNA genes through the binding of other proteins in the SL1 complex to the promoter.

The genes for tRNAs, 5S rRNA and some of the small RNAs involved in splicing and protein transport are transcribed by Polymerase III. These genes are characterised by promoters that lie within, and not upstream of the transcribed sequence.

The process of termination of transcription was found out from experiments in prokaryotes in which nucleus is not bound by a membrane. Therefore, synthesis of proteins on ribosomes occurs simultaneously with synthesis of mRNA on DNA.

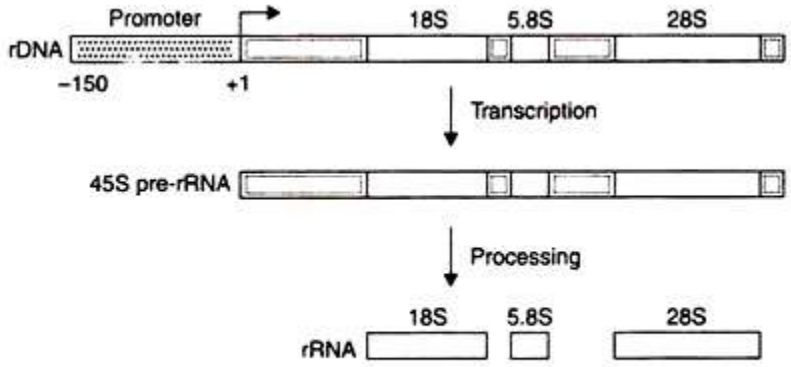


Fig. 15.9 The eukaryotic ribosomal RNA gene (rDNA) is transcribed into a large RNA molecule, the 45S pre-rRNA which is cleaved into the different rRNAs.

The main points related to transcription in eukaryotes are briefly discussed below:

1. Synthesis:

RNA is synthesized from a DNA template. The RNA is processed into messenger RNA [mRNA], which is then used for synthesis of a protein. The RNA thus synthesized is called messenger RNA (mRNA), because it carries a genetic message from the DNA to the protein-synthesizing machinery of the cell.

The main difference between RNA and DNA sequence is the presence of U, or uracil in RNA instead of the T, of thymine of DNA.

2. Template used:

The RNA is synthesized from a single strand or template of a DNA molecule. The stretch of DNA that is transcribed into an RNA molecule is called a transcription unit. A transcription unit codes the sequence that is translated into protein. It also directs and regulates protein synthesis.

The DNA strand which is used in RNA synthesis is called template strand; because it provides the template for ordering the sequence of nucleotides in an RNA transcript. The DNA strand which does not take part in DNA synthesis is called coding strand, because, its nucleotide sequence is the same as that of the newly created RNA transcript.

3. Enzyme Involved:

The process of transcription is catalysed by the specific enzyme called RNA polymerase. DNA sequence is enzymatically copied by RNA polymerase to produce a complementary nucleotide RNA strand. In eukaryotes, there are three classes of RNA polymerases: I, II and III which are involved in the transcription of all protein genes.

4. Genetic Information Copied:

In this process, the genetic information coded in DNA is copied into a molecule of RNA. The genetic information is transcribed or copied, from DNA to RNA. In other words, it results in the transfer of genetic information from DNA into RNA.

5. First Step:

The expression of a gene consists of two major steps, viz., transcription and translation. Thus transcription is the first step in the process of gene regulation or protein synthesis.

6. Direction of Synthesis:
As in DNA replication, RNA is synthesized in the 5′ —> 3′ direction. The DNA template strand is read 3′ –> 5′ by RNA polymerase and the new RNA strand is synthesized in the 5′ -> 3′ direction. RNA polymerase binds to the 3′ end of a gene (promoter) on the DNA template strand and travels toward the 5′ end.
The regulatory sequence that is before, or 5′, of the coding sequence is called 5′ un-translated region (5′ UTR), and sequence found following, or 3′, of the coding sequence is called 3′ un-translated region (3′ UTR). Transcription has some proofreading mechanisms, but they are fewer and less effective than the controls for copying DNA; therefore, transcription has a lower copying fidelity than DNA replication.

Mechanism of Transcription in Eukaryotes:
The mechanism of transcription consists of five major steps, viz:

(1) Pre-initiation,
(2) Initiation,
(3) Promoter clearance,
(4) Elongation and
(5) Termination.

These are briefly discussed as follows:
1. Pre-Initiation:
The initiation of transcription does not require a primer to start. RNA polymerase simply binds to the DNA and, along with other cofactors, unwinds the DNA to create an initiation bubble so that the RNA polymerase has access to the single-stranded DNA template. However, RNA Polymerase does require a promoter like sequence.

Proximal (core) Promoters:
TATA promoters are found around -30 bp to the start site of transcription. Not all genes have TATA box promoters and there exists TATA-less promoters as well. The TATA promoter consensus sequence is TATA(A/T)A(A/T).

2. Initiation:
In eukaryotes and archaea, transcription initiation is far more complex. The main difference is that eukaryotic polymerases do not recognize directly their core promoter sequences. In eukaryotes, a collection of proteins called transcription factors mediate the binding of RNA polymerase and the initiation of transcription.
Only after attachment of certain transcription factors to the promoter, the RNA polymerase binds to it. The complete assembly of transcription factors and RNA polymerase bind-to the promoter, called transcription initiation complex. Initiation starts as soon as the complex is opened and the first phosphodiester bond is formed. This is the end of Initiation.
RNA Pol II does not contain a subunit similar to the prokaryotic factor, which can recognize the promoter and unwind the DNA double helix. In eukaryotes, these two functions are carried out by a set of proteins called general transcription factors. The RNA Pol II is associated with six general transcription factors, designated as TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, where “TF” stands for “transcription factor” and “II” for the RNA Pol II.

TFIID consists of TBP (TATA-box binding protein) and TAFs (TBP associated factors). The role of TBP is to bind the core promoter. TAFs may assist TBP in this process. In human cells, TAFs are formed by 12 subunits. One of them, TAF250 (with molecular weight 250 kD), has the histone acetyltransferase activity, which can relieve the binding between DNA and histones in the nucleosome. The transcription factor which catalyses DNA melting is TFIIH. However, before TFIIH can unwind DNA, the RNA Pol II and at least five general transcription factors (TFIIA is not absolutely necessary) have to form a pre-initiation complex (PIC).

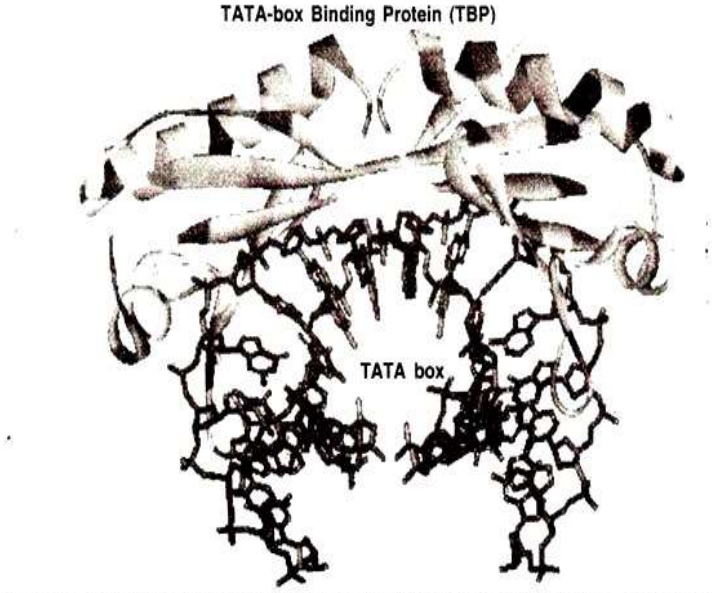


Fig. 22.1. Structure of the human TBP core domain complexed with DNA as determined by X-ray crystallography. The DNA includes the TATA element. PDB ID = 1CDW.

3. Promoter Clearance:

After the first bond is synthesized the RNA polymerase must clear the promoter. During this time there is a tendency to release the RNA transcript and produce truncated transcripts. This is called abortive initiation and is common for both Eukaryotes and Prokaryotes.

Once the transcript reaches approximately 23 nucleotides it no longer slips and elongation can occur. This is an ATP dependent process. Promoter clearance also coincides with Phosphorylation of serine 5 on the carboxy terminal domain which is phosphorylated by TFIIH.

4. Elongation:

For RNA synthesis, one strand of DNA known as the template strand or non-coding strand is used as a template. As transcription proceeds, RNA polymerase traverses the template strand and uses base pairing complementarity with the DNA template to create an RNA copy.

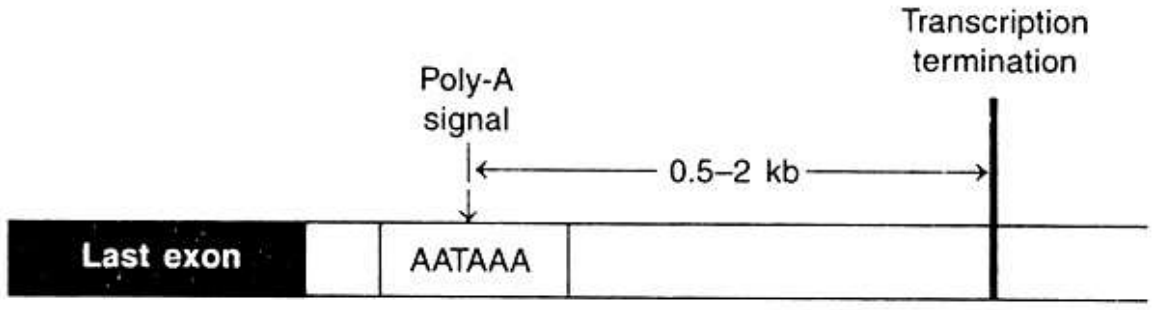
Although RNA polymerase traverses the template strand from 3′ —> 5′, the coding (non-template) strand is usually used as the reference point, so transcription is said to go from 5′ —> 3′.

This produces an RNA molecule from 5′ —> 3′, an exact copy of the coding strand (except that thymines are replaced with uracils, and the nucleotides are composed of a ribose (5-carbon) sugar where DNA has deoxyribose (one less oxygen atom) in its sugar-phosphate backbone).

After pre-initiation complex [PIC] is assembled at the promoter, TFIIH can use its helicase activity to unwind DNA. This requires energy released from ATP hydrolysis. The DNA melting starts from about -10 bp. Then, RNA Pol II uses nucleoside triphosphates (NTPs) to

synthesize a RNA transcript. During RNA elongation, TFIIF remains attached to the RNA polymerase, but all of the other transcription factors have dissociated from PIC.
The carboxyl-terminal domain (CTD) of the largest subunit of RNA Pol II is critical for elongation. In the initiation phase, CTD is un-phosphorylated, but during elongation it has to be phosphorylated. This domain contains many proline, serine and threonine residues.

5. Termination:
In eukaryotic transcription the mechanism of termination is not very clear. In other words, it is not well understood. It involves cleavage of the new transcript, followed by template-independent addition of As at its new 3′ end, in a process called polyadenylation.
Eukaryotic protein genes contain a poIy-A signal located downstream of the last exon. This signal is used to add a series of adenylate residues during RNA processing. Transcription often terminates at 0.5-2 kb downstream of the poly-A signal.



Transcription Factories in Eukaryotes:
Active transcription units that are clustered in the nucleus, in discrete sites are called ‘transcription factories’. Such sites could be visualized after allowing, engaged polymerases to extend their transcripts in tagged precursors (Br-UTP or Br-U), and immuno-labelling the tagged nascent RNA.
Transcription factories can also be localized using fluorescence in situ hybridization, or marked by antibodies directed against polymerases. There are ~10,000 factories in the nucleoplasm of a HeLa cell, among which are ~8,000 polymerase II factories and ~2,000 polymerase III factories. Each polymerase II factory contains ~8 polymerases.
As most active transcription units are associated with only one polymerase, each factory will be associated with ~8 different transcription units. These units might be associated through promoters and/or enhancers, with loops forming a ‘cloud’ around the factory.

Reverse Transcription in Eukaryotes:
Synthesis of DNA from RNA molecule in the presence of enzyme reverse transcriptase is referred to as reverse transcription. Reverse transcription was first reported by Temin and Baltimore in 1970 for which they were awarded Nobel prize in 1975. Reverse transcription is also known as Teminism. Some viruses (such as HIV, the cause of AIDS), have the ability to transcribe RNA into DNA.
In some eukaryotic cells, an enzyme is found with reverse transcription activity. It is called telomerase. Telomerase is a reverse transcriptase that lengthens the ends of linear chromosomes. Telomerase carries an RNA template from which it synthesizes DNA repeating sequence, or “junk” DNA. This repeated sequence of “junk” DNA is important because every time a linear chromosome is duplicated, it is shortened in length.

With “junk” DNA at the ends of chromosomes, the shortening eliminates some repeated, or junk sequence, rather than the protein-encoding DNA sequence that is further away from the chromosome ends.
Telomerase is often activated in cancer cells to enable cancer cells to duplicate their genomes without losing important protein-coding DNA sequence. Activation of telomerase can be part of the process that allows cancer cells to become immortal.

Role of Transcription Factors in Eukaryotes*:*
In eukaryotes, the association between DNA and histones prevents access of the polymerase and general transcription factors to the promoter. Histone acetylation catalys ed by HATs can relieve the binding between DNA and histones. Although a subunit of TFIID (TAF250 in human) has the HAT activity, participation of other HATs can make transcription more efficient. The following rules apply to most (but not all).
(i) Binding of activators to the enhancer element recruits HATs to relieve association between histones and DNA, thereby enhancing transcription.
(ii) Binding of repressors to the silencer element recruits histone deacetylases (denoted by HDs or HDACs) to tighten association between histones and DNA.

Regulation of transcription factor activity:
The term **transcription factor** is broadly used to describe proteins that influence the ability of RNA polymerase to transcribe a given gene. Such transcription factors can regulate the binding of the transcriptional apparatus to the core promoter and/or control the switch from the initiation to the elongation stage of transcription. Two categories of transcription factors play a key role in these processes. General transcription factors (GTF) are required for the binding of RNA polymerase to the core promoter and its progression to the elongation stage.
General transcription factors are necessary for a basal level of transcription. In addition, eukaryotic cells possess a diverse array of regulatory transcription factors that serve to regulate the rate of transcription of target genes. The importance of transcription factors
is underscored by the number of genes that encode this category of proteins. Regulatory transcription factors exert their effects by influencing the ability of RNA polymerase to begin transcription of a particular gene. They typically recognize *cis*-acting elements that are located in the vicinity of the core promoter. These DNA sequences are analogous to the operator sites found near bacterial promoters. In eukaryotes, these DNA sequences are generally known as control elements, or regulatory elements**.** When a regulatory transcription factor binds to a regulatory element, it affects the transcription of an associated gene. For example, the binding of regulatory transcription factors may enhance the rate of transcription. Such a transcription factor is termed an activator**,** and the sequence it binds to is called an enhancer**.** Alternatively, regulatory transcription factors may act as repressors by binding to elements called silencers and preventing transcription from occurring.
By studying transcriptional regulation, researchers have discovered that most eukaryotic genes, particularly those found in multicellular species, are regulated by many factors. This phenomenon is called combinatorial control because the combination of many factors determines the expression of any given gene.

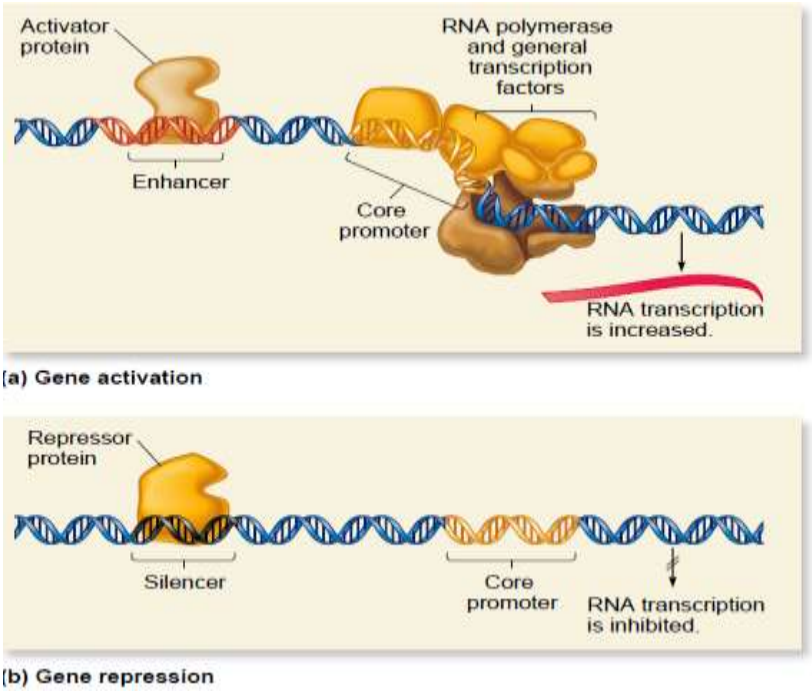


Fig 13: Overview of transcriptional regulation by regulatory transcription factors

At the level of transcription, the following are common factors that contribute to combinatorial control:

1. One or more activator proteins may stimulate the ability of RNA polymerase to initiate transcription.
2. One or more repressor proteins may inhibit the ability of RNA polymerase to initiate transcription.
3. The function of activators and repressors may be modulated in a variety of ways, including the binding of small effector molecules, protein–protein interactions, and covalent modifications.
4. Regulatory proteins may alter the composition or arrangements of nucleosomes in the vicinity of a promoter, thereby affecting transcription
5. DNA methylation may inhibit transcription, either by preventing the binding of an activator protein or by recruiting proteins that cause the chromatin to become more compact.

All five of these factors can contribute to the regulation of a single gene, or possibly only three or four will play a role. In most cases, transcriptional regulation is aimed at controlling the initiation of transcription at the promoter. The functions of the regulatory transcription factors themselves must also be modulated.

The genes they control must be turned on at the proper time, in the correct cell type, and under the appropriate environmental conditions. Therefore, eukaryotes have evolved different ways to modulate the functions of these proteins. The functions of regulatory transcription factor proteins are controlled in three common ways: through (1) the binding of a small effector molecule, (2) protein-protein interactions, and (3) covalent modifications.

Usually, one or more of these modulating effects are important in determining whether a transcription factor can bind to the DNA or influence transcription by RNA polymerase. For example, a small effector molecule may bind to a regulatory transcription factor and promote its binding to DNA.

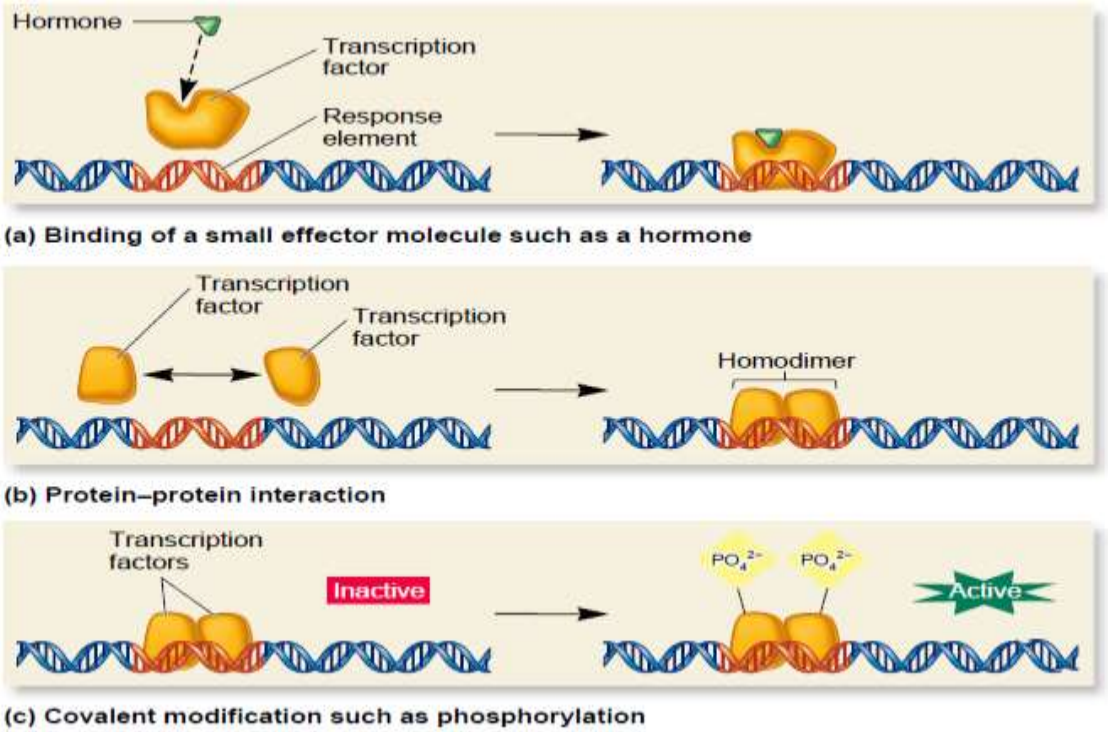


Fig 14: Mechanism of modulating regulatory transcription factor

Genes that encode general and regulatory transcription factor proteins have been identified and sequenced from a wide variety of eukaryotic species, including yeast, plants, and animals. Several different families of evolutionarily related transcription factors have been discovered. In recent years, the molecular structures of transcription factor proteins have become an area of intense research. Transcription factor proteins contain regions, called domains**,** that have specific functions. For example, one domain of a transcription factor may have a DNA-binding function, and another may provide a binding site for a small effector molecule. When a domain or portion of a domain has a very similar structure in many different proteins, such a structure is called a motif. The protein secondary structure known as an α helix is frequently found in transcription factors. The α helix is the proper width to bind into the major groove of the DNA double helix. In helix-turn-helix and helix-loop-helix motifs, an α helix called the recognition helix makes contact with and recognizes a base sequence along the major groove of the DNA Major groove is a region of the DNA double helix where the bases contact the surrounding water in the cell. Hydrogen bonding between an α helix and nucleotide bases is one way that a transcription factor can bind to a specific DNA sequence. In addition, the recognition helix often contains many positively charged amino acids (e.g., arginine and lysine) that favourably interact with the DNA backbone, which is negatively charged. Such basic domains are a common feature of many DNA-binding proteins.

Transcriptional gene regulation in eukaryotes:

Different mechanisms have been discovered that explain how a regulatory transcription factor can bind to a regulatory element and thereby affect gene transcription. For most genes, more than one mechanism is involved. The net effect of a regulatory transcription factor is to influence the ability of RNA polymerase to transcribe a given gene. However, most regulatory transcription factors do not bind directly to RNA polymerase.

For structural genes in eukaryotes, regulatory transcription factors commonly influence the function of RNA polymerase II by interacting with other proteins that directly bind to RNA polymerase II. Two protein complexes that communicate the effects of regulatory transcription factors are TFIID and mediator.

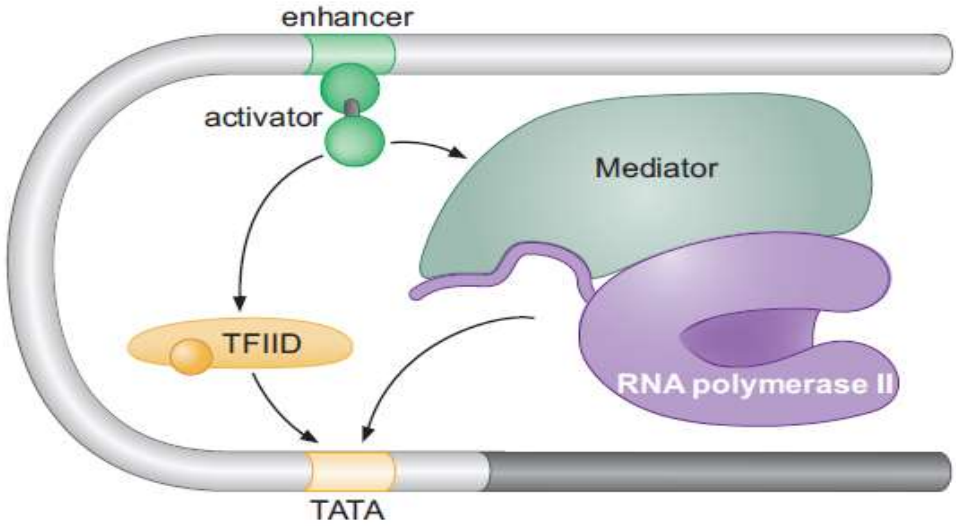


Fig 15. Activation transcription initiation by recruiting transcription machinery

Some regulatory transcription factors bind to a regulatory element and then influence the function of TFIID. TFIID is a general transcription factor that binds to the TATA box and is needed to recruit RNA polymerase II to the core promoter. Activator proteins are expected to enhance the ability of TFIID to initiate transcription. One possibility is that activator proteins could help recruit TFIID to the TATA box. Or they could enhance the function of TFIID in a way that facilitates its ability to bind RNA polymerase II. In some cases, activator proteins exert their effects by interacting with coactivators-proteins that increase the rate of transcription but do not directly bind to the DNA itself. In contrast, repressors inhibit the function of TFIID. They could exert their effects by preventing the binding of TFIID to the TATA box or by inhibiting the ability of TFIID to recruit RNA polymerase II to the core promoter.

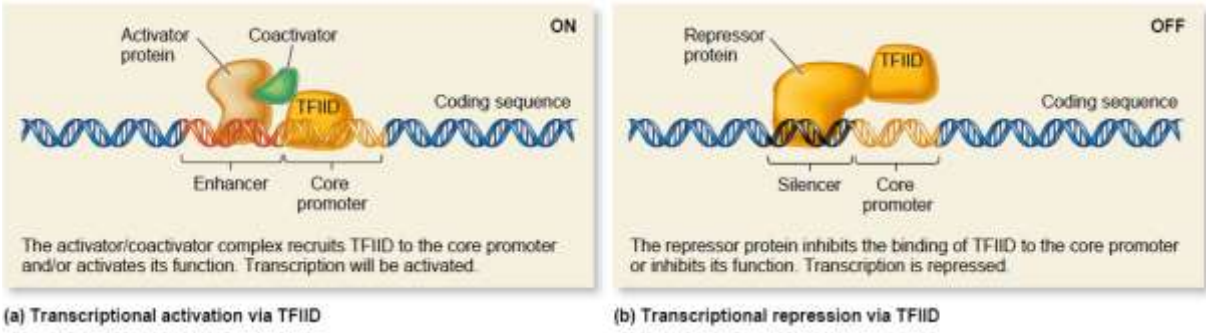


Fig 16. Transcription regulation via TFIID

A second way that regulatory transcription factors control RNA polymerase II is via mediator- a protein complex discovered by Roger Kornberg and colleagues in 1990. The term mediator refers to the observation that it mediates the interaction between RNA polymerase II and

regulatory transcription factors. Mediator controls the ability of RNA polymerase II to progress to the elongation stage of transcription. Transcriptional activators stimulate the ability of mediator to facilitate the switch between the initiation and elongation stages, whereas repressors have the opposite effect. An activator binds to a distant enhancer element. The activator protein and mediator are brought together by the formation of a loop within the intervening DNA.

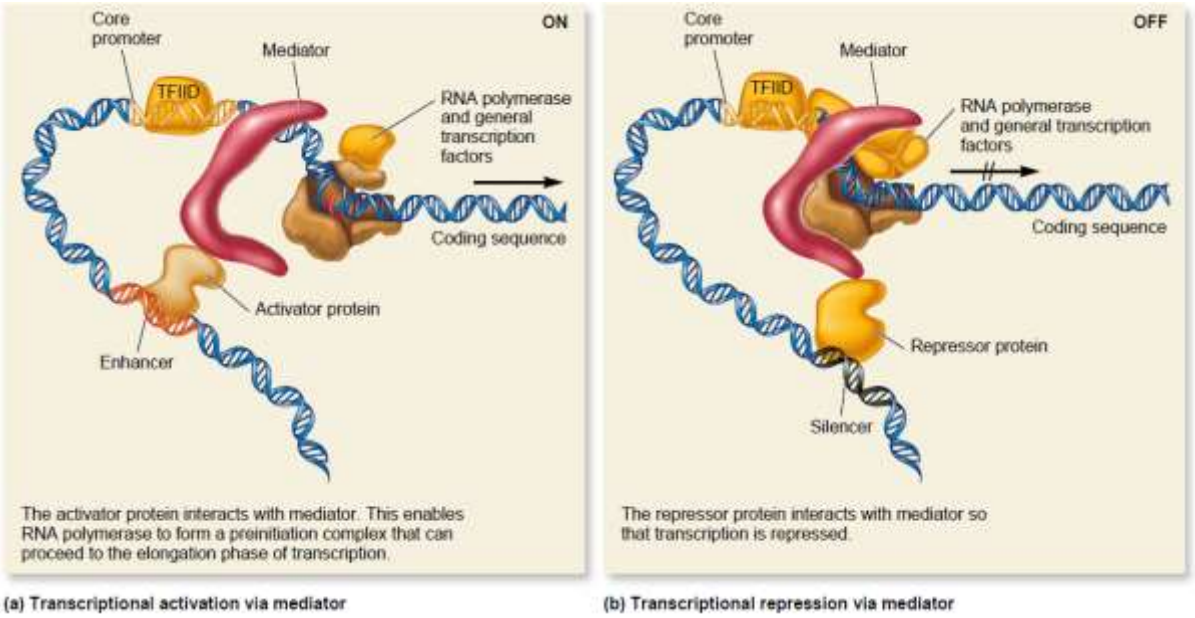


Fig 17: Effect of effector protein on mediator

A third way that regulatory transcription factors can influence transcription is by recruiting proteins to the promoter region that affect nucleosome positions and compositions. For example, certain transcriptional activators can recruit proteins to the promoter region that promote the conversion of chromatin from a closed to an open conformation.

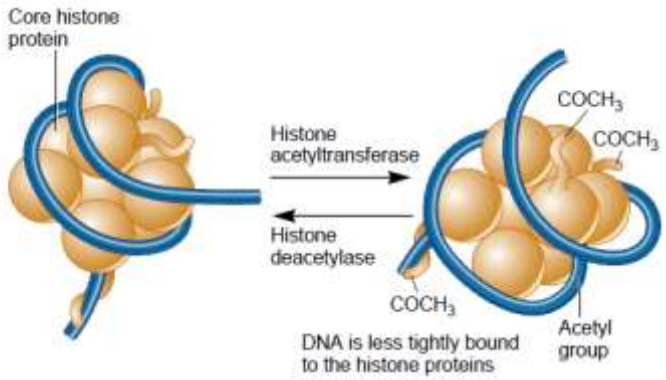


Fig 18: Effect of acetylation on nucleosome structure

Prokaryotic vs. Eukaryotic Transcription:

The process of transcription is the same in both eukaryotes and prokaryotes in several aspects. However, there are some differences in transcription of these two groups as highlighted below.

TABLE 21.2. Comparison of transcription in prokaryotes and eukaryotes

S.No.	Particulars	Prokaryotes	Eukaryotes
1.	Transcription and translation	Occur simultaneously	Occur seperately
2.	Process	Simple	More complicated
3.	mRNA	No need of modification	Needs modification
4.	Enzymes involved	RNA polymerase α, β, β′ and σ	RNA polymerases : I, II, and III
5.	Termination	Well known	Less clear
6.	Site	Cytoplasm	Nucleus
7.	DNA	Circular and free	Linear, packed with histones

1. The process is much more complicated in eukaryotes than prokaryotes.

2. In eukaryotes, transcription and translation take place separately in nucleus and cytoplasm respectively while in prokaryotes both processes take place simultaneously in the cytoplasm.

3. The eukaryotic mRNA contains introns and hence needs modification before taking part in protein synthesis. In prokaryote, the mRNA does not require modification.

4. Eukaryotes have DNA in the nucleus, whereas in prokaryotes DNA is in the cytoplasm.

Visualisation of Transcription:

The idea of visualizing gene transcription originally arose from light microscopic studies of lampbrush chromosomes in amphibian oocytes performed by Callan and Lloyd, and Gall in the 1960's; similar studies were done on the puffed polytene chromosomes of insects by Beermann and his colleagues. Oocyte-chromosomes are highly extended in the lampbrush state and contain thousands of chromosomal loci active in RNA synthesis.

Another favourable attribute of oocytes is that there is amplification (manifold increase) of rRNA genes during early oogenesis giving rise to hundreds of extra nucleoli in a nucleolus. In this system, transcription of ribosomal cistrons has been visualised.

During transcription on oocyte lampbrush chromosomes, the DNA in the condensed, beadlike chromomere unravels and is spun out into a loop and transcribed. The loop axis becomes covered by the transcribed RNA fibrils embedded in a protein matrix (Fig. 15.12). At the base of each RNP (ribonucleoprotein) fibril, an RNA polymerase molecule is attached. In male meiosis and somatic cells transcription produces fine hair-like outgrowths from the chromosomes (Fig. 15.13).

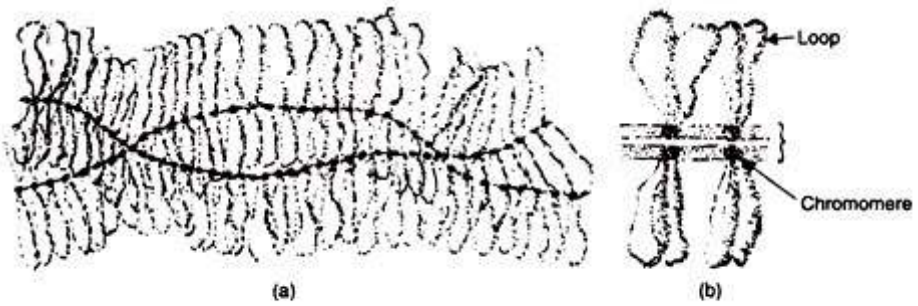


Fig. 15.12 (*a*) a portion from lampbrush chromosomes in amphibian oocytes and (*b*) schematic outline to show details of loop formation.

Fig. 15.13 The lampbrush state at meiotic prophase in the human male.

Puffing in the giant polytene chromosomes in the salivary glands of insects represents a direct way of correlating chromosome structure with gene transcription. Puff formation indicates genes that are actively transcribing RNA which would be translated into salivary proteins. Further work of Grossbach (1969) and others made it possible to relate the synthesis of a specific protein with a specific puff.

In 1969 Miller and Beatty developed a spreading technique for chromatin by which nascent RNP transcripts could be visualised in the electron microscope. The technique has since been applied to various materials. It allows us to visualize the spatial relationships between DNA, RNA polymerase and the RNA transcripts in situ.

EM studies have confirmed that most of the DNP (de-oxy-ribonucleoprotein) fibrils are entangled in the chromomeric mass and only a small proportion is extended into lateral loops. The initiation and termination sites for transcription are located at the two ends of the loop i.e., the thick and thin insertion sites of the loop. The lateral RNP fibrils which contain the nascent RNA transcripts are of increasing length. One loop is said to represent one transcriptional unit. Many times an active loop shows tandemly arranged transcription units separated by spacer regions (Fig. 15.14). The spacers represent non-transcribed regions. Up to 5 transcriptional units may be present on a loop; the loop is therefore a multi-gene structure.

These authors also found initiation sites of two transcriptional units overlapping each other. The drug actinomycin-D which inhibits transcription removes RNP fibrils from the template and causes loops to collapse. The initial products of transcription observed in EM are longer than the average hnRNA molecules isolated by biochemical techniques. Similar studies on transcription have also been conducted on interphase nuclei of somatic cells in some organisms.

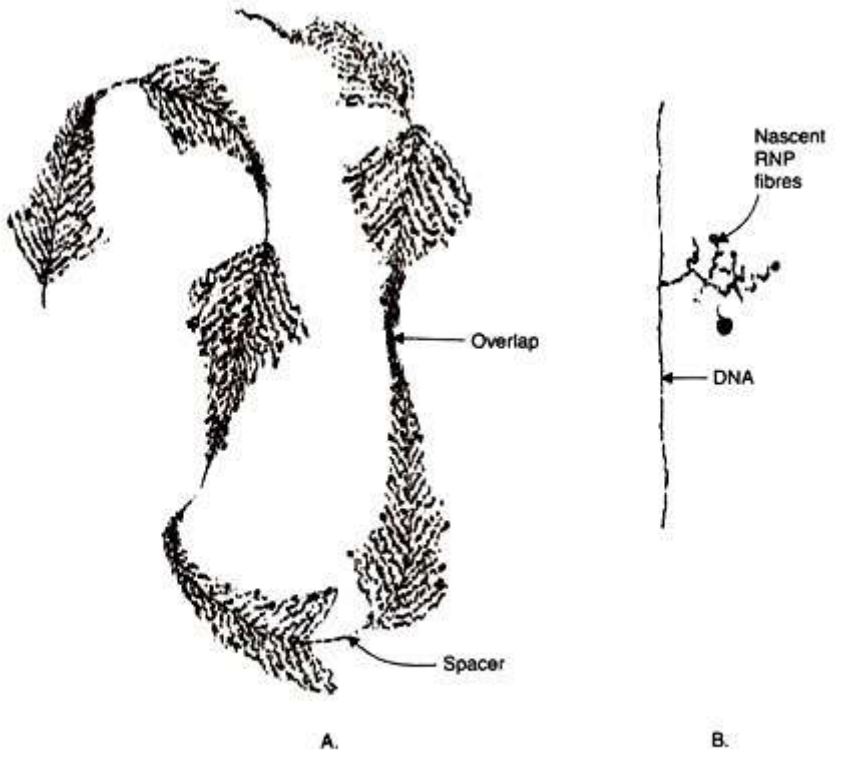


Fig. 15.14 Visualisation of transcribing genes. A—Transcription units are tandemly repeated on a DNA axis. Fig. A shows transcription of ribosomal cistrons and Fig. B exhibits non-ribosomal transcription.

Inhibitors of Transcription:

Several compounds can inhibit transcription of DNA by RNA polymerase. One group of compounds acts by binding non-covalently to the DNA template and modifying its structure; the other group binds to the RNA polymerase and inhibits its catalytic function. The most important inhibitor is actinomycin-D (AMD), an antibiotic produced by streptomyces.

Its phenoxazene ring intercalates between two GC pairs, while its two peptide side chains form H-bonds with guanine bases and project into minor groove of the double helix.

AMD does not interfere with the binding of RNA polymerase to DNA but inhibits chain elongation by preventing movement of core enzyme along the template. AMD does not interfere with replication of DNA. Aflatoxin, ethidium bromide and 2- acetyl-amino-fluorine also inhibit transcription by binding to DNA.

A group of bacterial antibiotics called rifamycins act by inhibiting bacterial RNA polymerases. One such compound known as rifampicin binds non-covalently to the β subunit of RNA polymerase so that chain initiation is inhibited, but does not affect chain elongation, α- amanitin blocks one of the RNA polymerase enzymes present in eukaryotic cells; but bacterial mitochondrial or chloroplast RNA polymerases are not affected by it.

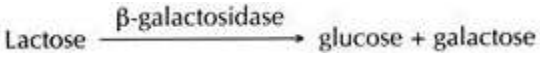
Gene Regulation in Prokaryotes:

Transcriptional Regulation of Gene Expression in Prokaryotes:

Gene transcription is regulated in bacteria through a complex of genes termed operon. These are transcriptional units in which several genes, with related functions, are regulated together. Other genes also occur in operons which encode regulatory proteins that control gene expression. Operons are classified as inducible or repressible.

Inducible and Repressible System:

The β galactosidase in E. coli is responsible for hydrolysis of lactose into glucose and galactose.



If lactose is not supplied to E. coli cells, the presence of β galactosidase is hardly detectable. But as soon as lactose is added, the production of β galactosidase enzyme increases. The enzyme falls as quickly as the substrate (lactose) is removed.
Such enzymes whose synthesis can be induced by adding the substrate are known as inducible enzymes and the genetic system responsible for the synthesis of such an enzyme is called inducible system. The substrate whose addition induces the synthesis of an enzyme is inducer. In some other cases, the situation is reverse. For instance, when no amino acids are supplied from outside, the E. coli cells can synthesize all the enzymes needed for the synthesis of different amino acids. However, if a particular amino acid, for instance, histidine, is added, the production of histidine synthesizing enzyme falls. In such a system, the addition of the end product of biosynthesis checks the synthesis of the enzymes needed for the biosynthesis. Such enzymes whose synthesis can be checked by the addition of the end product are repressible enzymes and the genetic system is known as repressible system. The end product, the addition of which check the synthesis of the enzyme is co-repressor.
A class of molecules called repressors are found in cells and these repressors check the activity of genes. An active repressor can be made inactive by adding inducer, while an inactive repressor can be made active by adding a co-repressor.

Operon Model:
A hypothesis to explain the induction and repression of enzyme synthesis was first proposed by Jacob and Monod. The scheme proposed by them is called Operon Model.

This consists of the components:
(i) Structural genes
(ii) Promoter genes
(iii) Operator genes
(iv) Regulator genes
(v) Effector or inducer

a. Structural Gene:
These are directly concerned with the synthesis of cellular proteins. They produce the mRNAs through transcription and determine the sequence of amino acids in the synthesized proteins. All the structural genes under an operon may form one long poiycistronic or polygenic mRNA molecule.

b. Operator Gene:
This is located adjacent to the structural gene. It determines whether the structural genes are to be repressed by the repressor protein, a product of regulator gene. The operator gene is the site of binding of the repressor protein, the latter binds to the operator forming an operator-repressor

complex. When the repressor binds to the operator, transcription of the structural genes cannot occur.

c. Regulator Gene:

These genes synthesize repressor. Repressor may be either an active repressor or an inactive repressor. Repressor protein has one active site for operator recognition and other active site for inducer. In absence of an inducer protein, the repressor binds to the operator gene and blocks the path of RNA polymerase. Thus the structural genes are unable to transcribe mRNA and consequently protein synthesis does not occur. n presence of an inducer, the repressor protein binds to the inducer to form an inducer-repressor complex. The repressor when binds with inducer undergoes a change and becomes ineffective and as a result it cannot bind to the operator gene and the protein synthesis is possible.

d. Promoter Gene:

The actual site of transcription initiation is known as promoter gene which lies to the left of the operator gene. It is believed that RNA polymerase binds to and moves from the promoter site.

e. Effector or Inducer:

Effector is a small molecule (sugar or amino acid) that can be linked to a regulator protein and will determine whether repressor will bind the operator or not. In the inducible operon, these effector molecules are called inducer. In repressible operon, these effector molecules are called co-repressor.

I. Inducible Operon:

Lac Operon:

The best known operon is the lac operon. In 1961, F. Jacob and J. Monod proposed the operon model to explain the genetic basis of enzyme induction and repression in prokaryotes.

A few years later (1965), these two investigators were awarded the Nobel Prize for their most incisive work. Although Jacob and Monod's original operon model applied specifically to the regulation of the genes for lactose metabolism in E. coli, additional findings by these two scientists as well as the work of many others have revealed the mechanisms by which other operons function.

The lac operon exercises both positive and negative control. Negative control is in the sense that the operon is normally **"on"** but is kept **"off"** by the regulator gene, i.e., the genes are not allowed to express unless required.

The lac repressor exercises negative control. Positive control is that in which the regulator gene will stimulate the production of the enzyme. Catabolite activator protein (CAP) facilitates transcription, so it exercises positive control. Two unique proteins are thus involved in the regulation of the lac operon which are lac repressor and CAP. Lactose is a disaccharide molecule. In order to utilize lactose as a carbon and energy source, the lactose molecules must be transported from the extracellular environment into the ceil, and then undergo hydrolysis into glucose and galactose. These reactions are catalysed by three enzymes. The lac operon consists of three structural genes (lac Z, Y, A) which code for these three enzymes (Fig. 17.2).

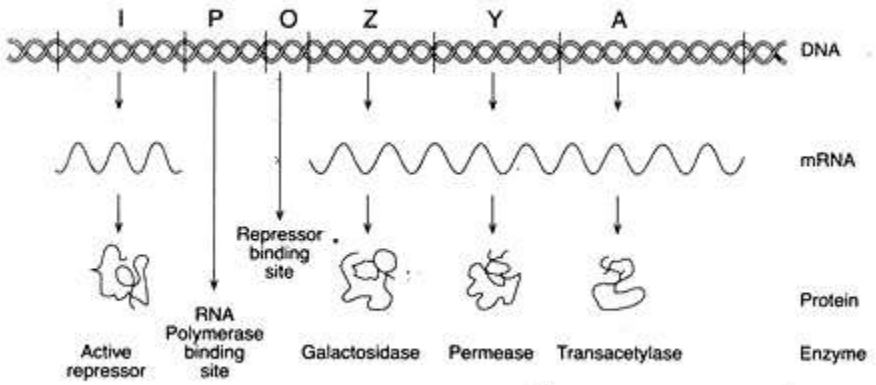


Fig. 17.2: The model of gene regulation of Jacob and Monod as applied to the lac operon in E. coli (from G. S. Stent)

The normal inducer of the lac operon is allolactose, which is produced from lactose by β-galactosidase (Fig. 11-13). A few copies of this enzyme are present in E. coli cells even in the uninduced state. When allolactose combines with the repressor, a steric change occurs that causes the repressor to be released from the operator. As a result, transcription of the structural genes by RNA polymerase begins and the three enzymes quickly appear in the cells.

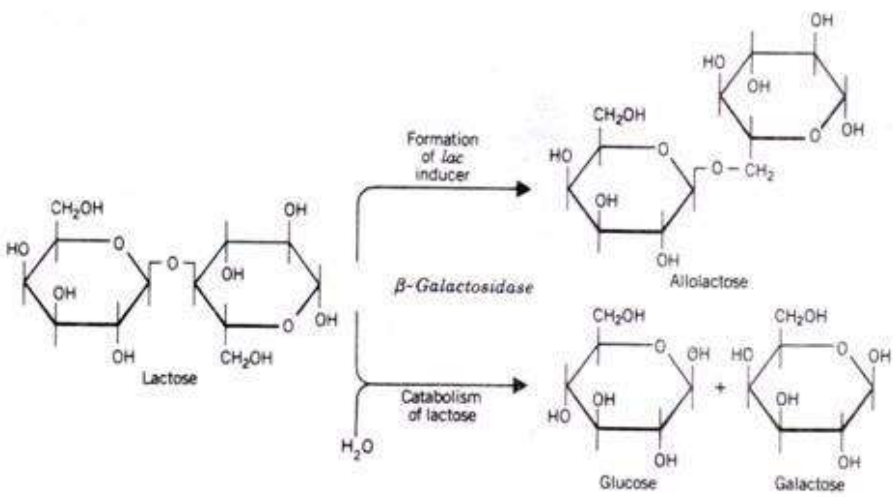


FIGURE 11–13 The two reactions that are catalyzed by the enzyme β-galactosidase. Allolactose produced in the upper reaction is an inducer of the lac operon.

lac Z gene — codes for enzyme β galactosidase which breaks lactose into galactose and glucose

lac Y gene — codes for permease which transports lactose into the cell

lac A gene — codes for transacetylase which transfer the acetyl group from acetyl CoA to galactose.

Negative Control of lac Operon:

lac repressor is synthesized through the activity of the lac I gene called the regulator gene. This repressor is an allosteric protein

(i) That can bind the lac DNA at the operator site, or

(ii) That can bind to inducer.

In the absence of inducer, DNA binding site of repressor is functional. The repressor protein binds to the DNA at the operator site of the lac locus and blocks the transcription of the lac genes by RNA polymerase. Thus lac enzyme synthesis is inhibited (Fig. 17.3A).

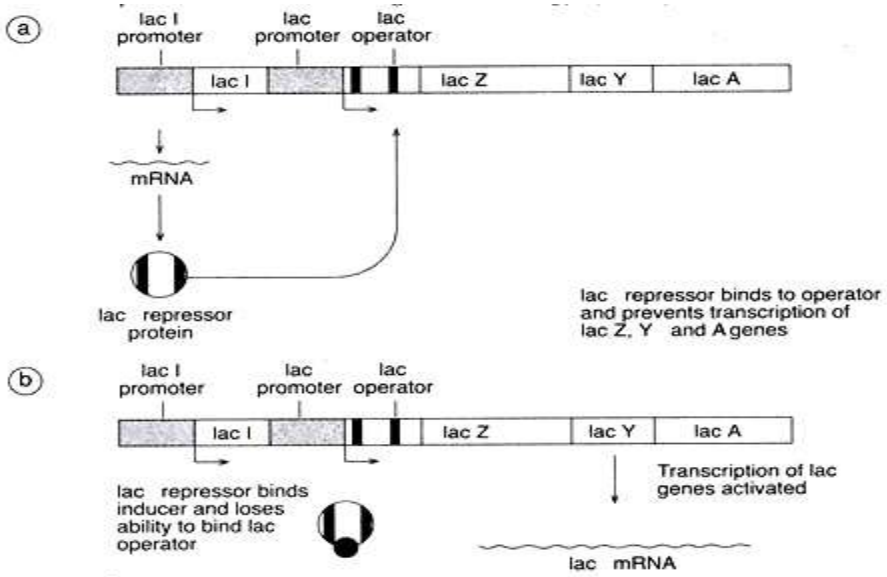


Fig. 17.3A: Regulation of lac operon (a) in the absence and (b) in the presence of inducer (after Winter, Hickey & Fletcher)

Lactose is not the real inducer of the lac operon. It binds to repressor to increase its affinity for operator. On the other hand, the bound protein of the inactive repressor is the allolactose. While β galactosidase breaks lactose into glucose and galactose, a side reaction changes galactose to allolactose and galactobiose.

This allolactose prevents the anti-inducing effect of lactose. When the allolactose (inducer) binds to the repressor, it changes the form of DNA binding site making the repressor inactive and release from- the operator site. Thus transcription of lac genes are possible.

Positive Control of lac Operon:

It is an additional regulatory mechanism which allows the lac operon to sense the presence of glucose, an alternative and preferred energy source to lactose. If glucose and lactose are both present, cells will use up the glucose first and will not utilize energy splitting lactose into its component sugars.

The presence of glucose in the cell switches off the lac operon by a mechanism called catabolite repression which involves a regulatory protein called the catabolite activator protein (CAP). CAP binds to a DNA sequence upstream of the lac promoter and enhances binding of the RNA polymerase and transcription of the operon is enhanced (Fig. 17.3B).

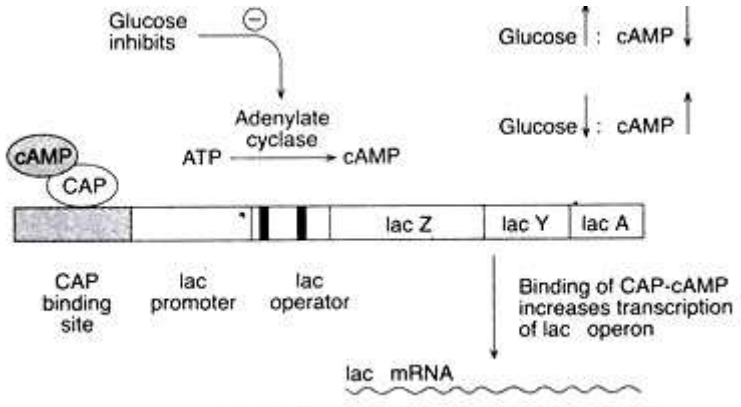


Fig. 17.3B: Catabolite repression of lac operon

CAP only binds in the presence of a derivative of ATP called cyclic adenosine monophosphate (cAMP) whose levels are influenced by glucose. The enzyme adenylate cyclase catalyses the formation of cAMP and is inhibited by glucose. When glucose is available to the cell, adenylate cyclase is inhibited and cAMP levels are low.

Under these conditions CAP does not bind upstream of the promoter and the lac operon is transcribed at a very low level. Conversely, when glucose is low, adenylate cyclase is not inhibited, cAMP is higher and CAP binds increasing the level of transcription from the operon. If glucose and lactose are present together, the lac operon will only be transcribed at a low level. However when the glucose is used up, catabolite repression will end and transcription from the lac operon increases allowing the available lactose to be used up.

Catabolic Repression:

Catabolic repression is a specific type of repression of enzyme production in which a metabolite such as glucose acts to repress the formation of enzymes that would allow the catabolism of other, related metabolites. For example, when E. coli cells are cultured in a medium that is rich in glucose, the glucose represses the formation of β-galactosidase even if lactose (an inducer of this enzyme) is added to the medium. Glucose is even known to repress the production of enzymes that formerly were thought to be constitutive.

A common phenomenon in bacteria is the suppression of aerobic respiration and electron transport by high glucose concentrations, even in the presence of ample oxygen. Under this condition, the cells utilize the glycolytic and fermentative pathways. The manner in which the catabolite brings about the effect is only partially understood. In the case of catabolite repression by glucose in bacteria, it appears that glucose affects the amount of cyclic AMP (cAMP) present in the cells.

When the concentration of glucose is high, the concentration of cAMP is low; and low levels of glucose are accompanied by high concentrations of cAMP. It is possible that glucose affects the synthesis of cAMP, which is formed from ATP by adenyl cyclase (Fig. 11-14).

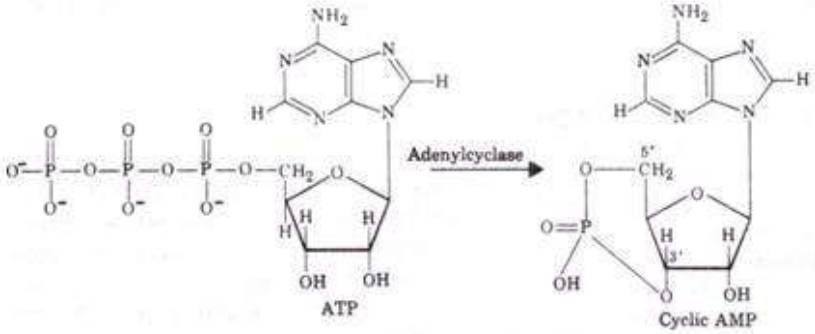


FIGURE 11–14 Formation of cyclic AMP from ATP by the enzyme, *adenylcyclase*.

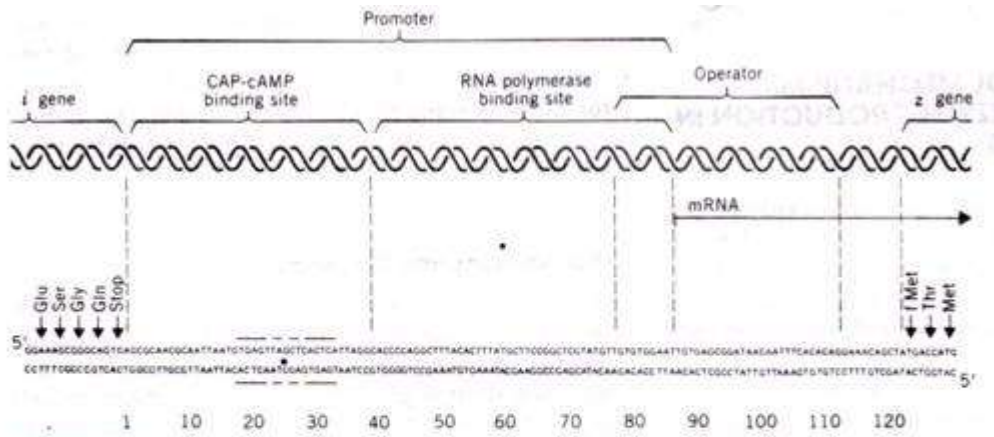


FIGURE 11–15 Organization and nucleotide sequences of the *lac* operon. The promoter consists of two components: the site that binds the cAMP–CAP complex and the RNA polymerase binding site. The adjoining segments of the repressor (*i*) and *β-galactosidase* (*z*) structural genes are also shown. The horizontal arrow (labeled mRNA) shows the position at which the transcription of the operon begins (i.e., what will be the 5′ end of the mRNA). The numbers at the bottom of the figure give the distances (in base pairs) from the end of the *i* gene. (From Gardner and Snustad, *Principles of Genetics*, 7th ed., p. 389 Copyright © 1984 by John Wiley & Sons, Inc.)

cAMP is necessary for the CAP to bind to the promoter site. cAMP binds to the CAP, and once the cAMP-CAP complex binds to the promoter (Fig. 11-15), the RNA polymerase can attach to the promoter and begin transcription. For example, in the case of the lac operon, when the glucose level is high (even if lactose is also present), the cAMP is low, and therefore the cAMP—CAP complex is not available to bind to the promoter and allow transcription to start. However, in the absence of glucose and in the presence of lactose (which forms a complex with the repressor), cAMP is plentiful and is available to combine with the CAP so that transcription proceeds. The rate of lac operon transcription in the absence of glucose is 50 times as great as in the presence of glucose.

II. Repressible Operon:

Trp Operon:

The trp operon consists of the following components:

(i) Structural genes (trp E, D, C, B and A):

This operon contains five structural genes encoding enzymes involved in biosynthesis of the amino acid tryptophan. The genes are expressed as a single mRNA transcribed from an upstream promoter.

(ii) Promoter gene (trp P):

It is the promoter region which is the binding site for RNA polymerase.

(iii) Operator gene (trp O):

It is the operator region which binds with the repressor.

(iv) Leader gene (trp L):
It is the leader region which is made of 162 nucleotides prior to the first structural gene trp E. It has four regions, region 1 has the codon for tryptophan, region 2, 3 and 4 regulate the mRNA synthesis of the structural genes.

Expression of the operon is regulated by the level of tryptophan in the cell (Fig. 17.4). A regulatory gene upstream of the trp operon encodes a protein called the trp repressor. This protein binds a DNA sequence called the trp operator which lies just downstream of the trp promoter partly overlapping it.

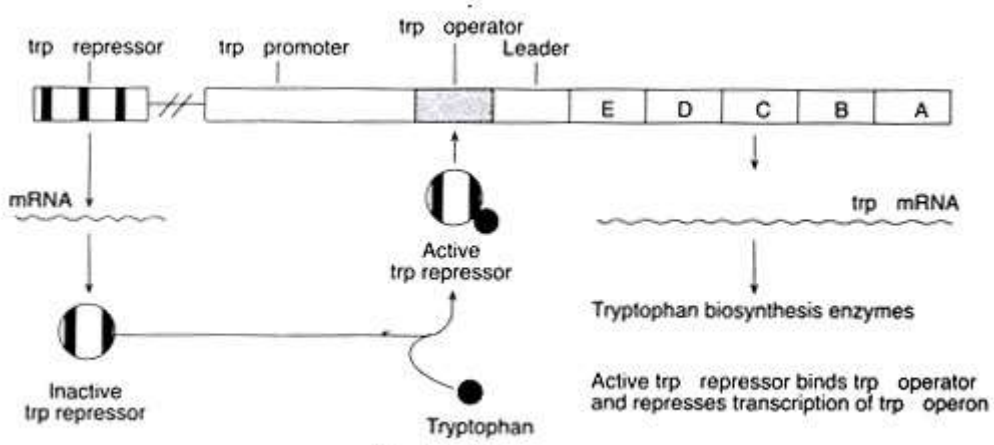


Fig. 17.4: The trp operon

When tryptophan is present in the cell it binds to the trp repressor protein enabling it to bind the trp operator sequence, obstructing binding of the RNA polymerase to the trp promoter and preventing transcription of the operon.
In the absence of tryptophan, the trp repressor is incapable of binding the trp operator and transcription of the operon proceeds. Tryptophan, the end product of the enzymes encoded by the trp operon, thus acts as a co-repressor with the trp repressor protein and inhibits its own synthesis by end product inhibition.

Attenuation:
Attenuation is an alternative regulatory mechanism that allows fine adjustment of expression of the trp operon and other operons (phe, his, leu, thr operon). The transcribed mRNA sequence between the trp promoter and the first trp gene are capable of forming either a large stem-loop structure that does not influence transcription or a smaller stem loop which acts as transcription terminator (Fig. 17.5).
The relative position of the sequences does not allow the formation of both stem-loops at a time. Attenuation depends on the fact that transcription and translation are linked, i.e., ribosomes attach to mRNAs as they are being transcribed and begin translating them into protein. Binding of ribosomes to the trp mRNA influences which of the two stem-loops can form and so determines whether termination occurs or not (Fig. 17.5).
A short coding region upstream of the stem-loop region contains tryptophan codons which is translated before the structural genes. When tryptophan levels are adequate, RNA polymerase transcribes the leader region closely followed by a ribosome which prevents formation of the larger stem-loop, allowing the terminator loop to form ending transcription. If tryptophan is

lacking, transcription is initiated, but not subsequently terminated because the ribosome is stalled, the RNA polymerase moves ahead and the large stem-loop forms. Formation of the terminator loop is blocked and transcription of the operon proceeds. When tryptophan present at intermediate levels, some transcripts will terminate and others not.

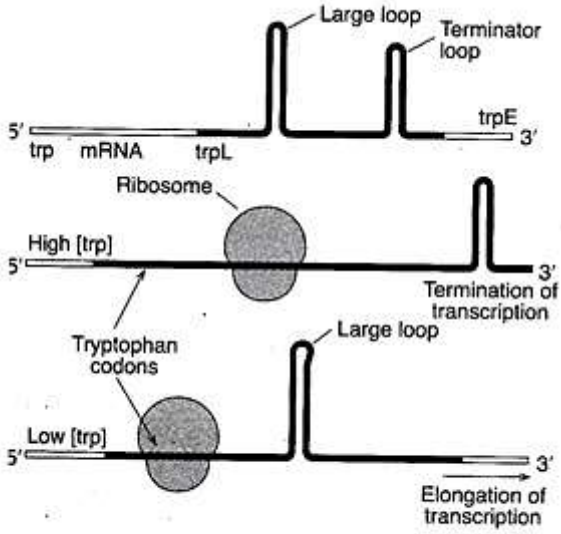


Fig. 17.5A: Transcriptional attenuation in the trp operon (from Winter, Hickey, Fletcher)

Attenuation thus allows the cell to synthesize tryptophan according to its exact requirements. Overall, the trp repressor determines whether the operon is switched on or off and attenuation determines how efficiently it is transcribed. The sequence of the mRNA suggests that ribosome stalling influences termination at the attenuator. The ability of the ribosome to proceed through the leader region may control transition between these structures. The structure determines whether the mRNA can provide the features needed for termination or not.

When tryptophan is present, ribosomes are able to synthesize the leader peptide. They will continue along the leader section of the mRNA to the UGA codon, which lies between regions 1 and 2. By progressing to this point, the ribosomes extend over region 2 and prevent it from base pairing. The result is that region 3 is available to base pair with region 4, generating the terminator hairpin. Under these conditions, therefore, RNA polymerase terminates at the attenuator.

However, when there is no tryptophan, ribosomes initiate translation of the leader peptide but stall at the trp codons which is at the region 1. Thus the region 1 cannot base pair with region 2. If this happens, even while the mRNA itself is being synthesized, region 2 and 3 will be base- paired before region 4 has been transcribed. This compels region 4 to remain in a single stranded form. In the absence of the terminator hairpin, RNA polymerase continues transcription past the attenuator.

The two operon models described above can be summarized as given below:

(i) Inducible System:

Active Repressor + Operator → System OFF

Active Repressor + Inducer = Inactive Repressor → System ON

(ii) Repressible System:

Apo-repressor and co-repressor complex = Active repressor → System OFF
Apo-repressor = Inactive Repressor → System ON

Importance of Gene Regulation:

1. There are two types of gene action – constitutive and regulated.
2. The constitutive gene action occurs in those systems which operate all the time and the cell cannot live without them, e.g., glycolysis. It does not require repression. Therefore, regulator and operator genes are not associated with it.
3. In regulated gene action all the genes required for a multistep reaction can be switched on or off simultaneously.
4. The genes are switched on or off in response to particular chemicals whether required for metabolism or are formed at the end of a metabolic pathway.
5. Gene regulation is required for growth, division and differentiation of cells. It brings about morphogenesis.

Probable Questions:

1. Write down the composition of RNA polymerase of E.coli. What is sigma factor.
2. Write down the role of consensus sequences in initiation of transcription.
3. Discuss rho dependent and rho independent termination of transcription.
4. What is reverse transcription ? describe different step of reverse transcription.
5. Write down the differences between transcription and reverse transcription.
6. How transcription is initiated in Eukaryotes?
7. What is promoter clearance?
8. How transcription factors regulate transcription in eukaryotes?
9. Differentiate prokaryotic and eukaryotic transcription.
10. Write short note on inhibition of transcription.
11. Define inducible and repressible system? Give examples.
12. Describe different components of an operon.
13. Write down the structural components of Lac operon.
14. How allolactose induce lac operon? Explain with suitable diagram.
15. Describe positive role of lac operon.
16. What is catabolic repression? Explain.
17. Describe structural components of trp operon.
18. How attenuation controls trp operon.
19. Describe the significance of gene regulation.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-II

Post-transcriptional gene control - types of introns and their splicing, evolution of introns, catalytic RNA, alternative splicing and proteome diversity, regulation of Pre-mRNA Processing, micro RNA and other non-coding RNAs, degradation of RNA

Objective: In this unit we will discuss about different types of post transcriptional mechanisms and their regulations. We will also discuss about catalytic RNA and different types of non coding RNA including micro RNA. Alternative splicing and their role in protein diversity generation will also be discussed.

Introduction:

Almost all types of RNA molecules undergo post synthesis transformation which is called RNA processing. Prokaryotic mRNA is generally not processed. In prokaryotes 5′-end of prokaryotic mRNA starts translation while the 3′-end is still under synthesis. Eukaryotic mRNA undergoes maximum processing. Both prokaryotic and eukaryotic tRNAs and rRNAs undergo processing. Except prokaryotic mRNA, all other kinds of RNA are processed immediately after synthesis.

Processing of Eukaryotic mRNA:
Newly synthesized mRNA is called primary transcription or precursor mRNA. It is quite different from the mRNA that takes part in protein synthesis. Large-scale changes take place in precursor mRNA. These changes are called processing of mRNA. Both 5′-end 3′-end of mRNA are modified. Non-coding regions are removed by splicing. The changes lead to the formation of mature mRNA which takes part in protein synthesis.

There are 4 main process by which eukaryotic RNA are processed: These are described below:

A. 5' Methyl Guanosine Capping:
Eukaryotic pre-mRNAs are altered at their 5′ end by a modification known as capping which involves addition of the modified nucleotide, 7-methylguanosine. The cap is added by the enzyme guanyl transferase which joins GTP by an unusual 5′—- > 5′ triphosphate linkage to the first nucleotide of the mRNA.
Methyl transferase enzymes then add a -CH_3 group to the 7-nitrogen of the guanine ring and, usually, to the 2′ hydroxyl group on the ribose sugar of the next two nucleotides. Capping protects the mRNA from being degraded from the 5′ end by exonucleases in the cytoplasm and is also a signal allowing the ribosome to recognize the start of a mRNA molecule (Fig. 16.9A).

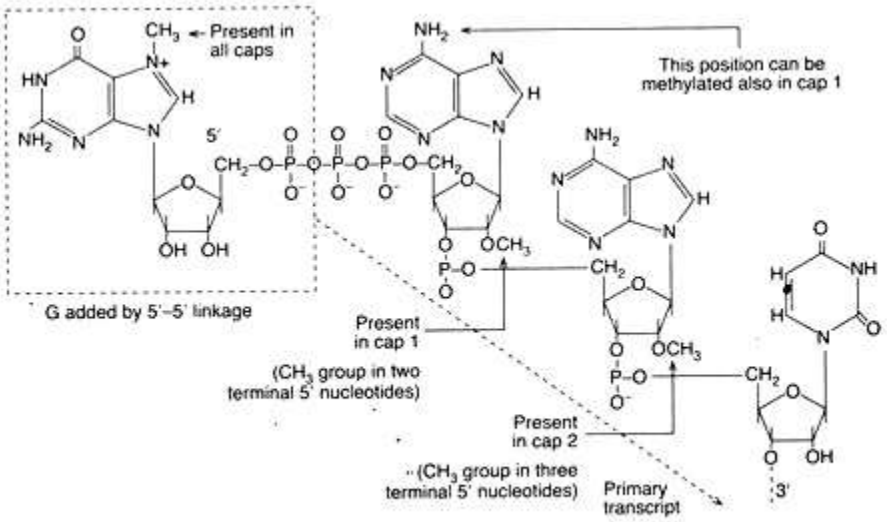


Fig. 16.9A: Capping of 5' end of mRNA by cap0, cap1 and cap2 due to methylation at several positions (from P. K. Gupta)

B. Polyadenylation:

Most eukaryotic pre- mRNA are modified at their 3′ ends by the addition of a sequence of up to 250 adenines, known as a poly A tail. This modification is called polyadenylation and requires the presence of signal sequences in the pre-mRNA.

These consist of the polyadenylation signal sequence, 5′ AAUAAA 3′, which occurs near the 3′ end of the pre-mRNA. The sequence YA (Y = pyrimidine) occurs in the next 11-20 bases and a GU rich sequence is often present further downstream. A number of specific proteins recognize and bind these signal sequences forming a complex which cleaves the mRNA about 20 nucleotides downstream of the 5′ AAUAAA 3′ sequence. The enzyme poly(A) polymerase then adds adenines to the 3′ end of the molecule. The purpose of the poly A tail may be to protect the mRNA from degradation of the coding sequence at the 3′ end by exonucleases (Fig. 16.9B).

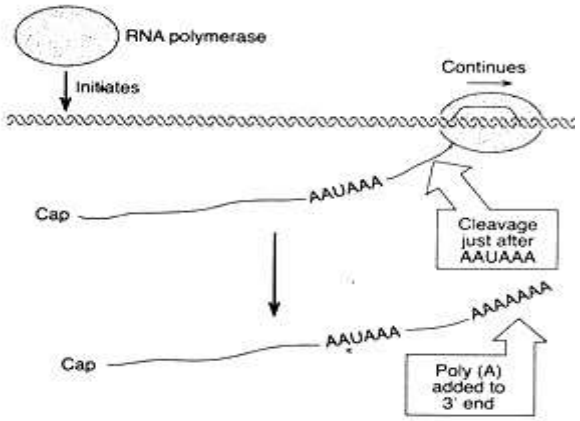


Fig. 16.9B: Cleavage at AAUAAA and polyadenylation at the cleaved 3' end

C. Intron Splicing :

Till recently it was believed that coding sequence of DNA and amino acids of polypeptide is collinear. The coding sequences are continuous and codon for one amino acid is adjacent to the codon for the next amino acid. The open reading frame is a single stretch of codons without any gap.

But now it has been discovered that coding sequence of most of eukaryotic genes is split into stretches of codons interrupted by stretches of non-coding sequences. Most human genes are discontinuous. The coding sequences of DNA of the gene are called exons. In between exons, there are intervening non-coding sequences called introns. This type of genes are called split genes or interrupted genes. They are most common in eukaryotes. They are also found in viruses but rarely in bacteria.

The terms exons and introns were given by Gilbert in 1977. It was discovered in Amphibia, mammals and some other animals that genes are not represented by continuous sequence of nucleotides. Introns are removed by excision and discarded.

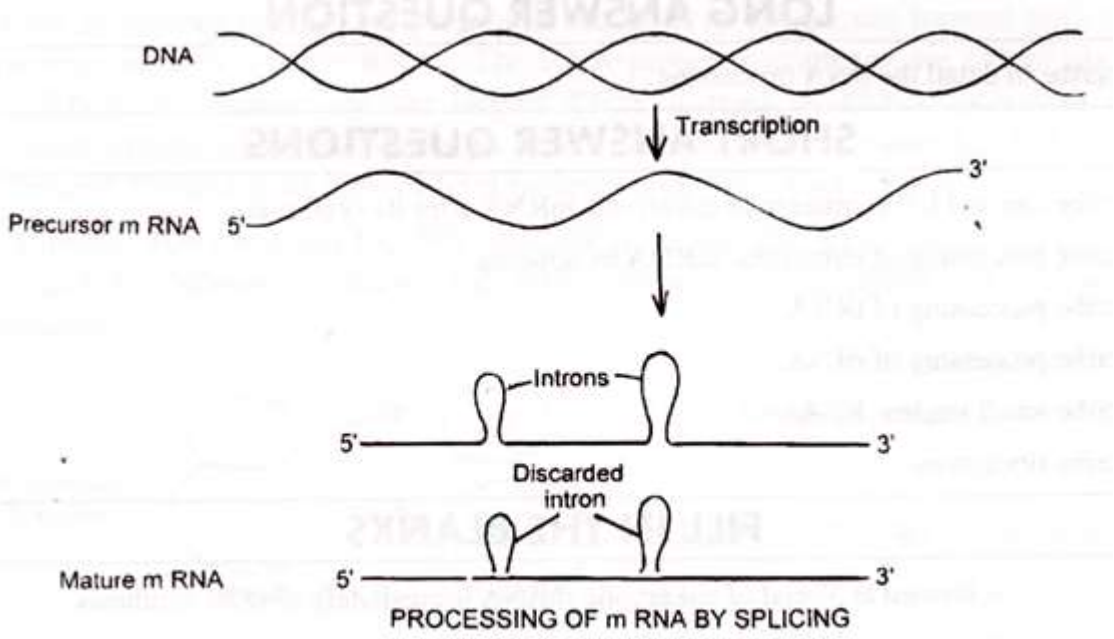


Fig. 9.1. Processing of mRNA by Splicing

The size of introns and exons varies Introns are usually much larger than the exons. A typical exon consists of small number of nucleotides whereas an intron may consist of thousands of nucleotides. Moreover, the introns constitute a large portion of the genome.

During the processing of mRNA in eukaryotes, the amount of discarded RNA ranges from 50-90 percent of the primary transcript. The remaining segments of mRNA or exons are joined together to form the finished mature mRNA. The split genes are transcribed into a single mRNA copy of the entire gene, called primary transcript. It consists of both exons and introns. The removal of introns by excision is called RNA splicing. The 5′-segment or the cap and the 3′-segment of the trailer are never discarded. Some examples of number of introns are — a-globin has two introns. Yeast genes have one intron, ovalbumin has seven, a-collagen has 52 introns and Titin gene of human beings has 363 introns.

Translation takes place only after the splicing is completed.

Phillip Sharp and Richard Robert won Nobel Prize for medicine in 1993 for their work on split genes. They studied the hybrid of adenovirus mRNA and one (template) strand of DNA under the electrons microscope. The mRNA was found to hybridize with discontinuous stretches of

genomic DNA. The intervening stretches of DNA were in the form of loops. These loops represented introns.

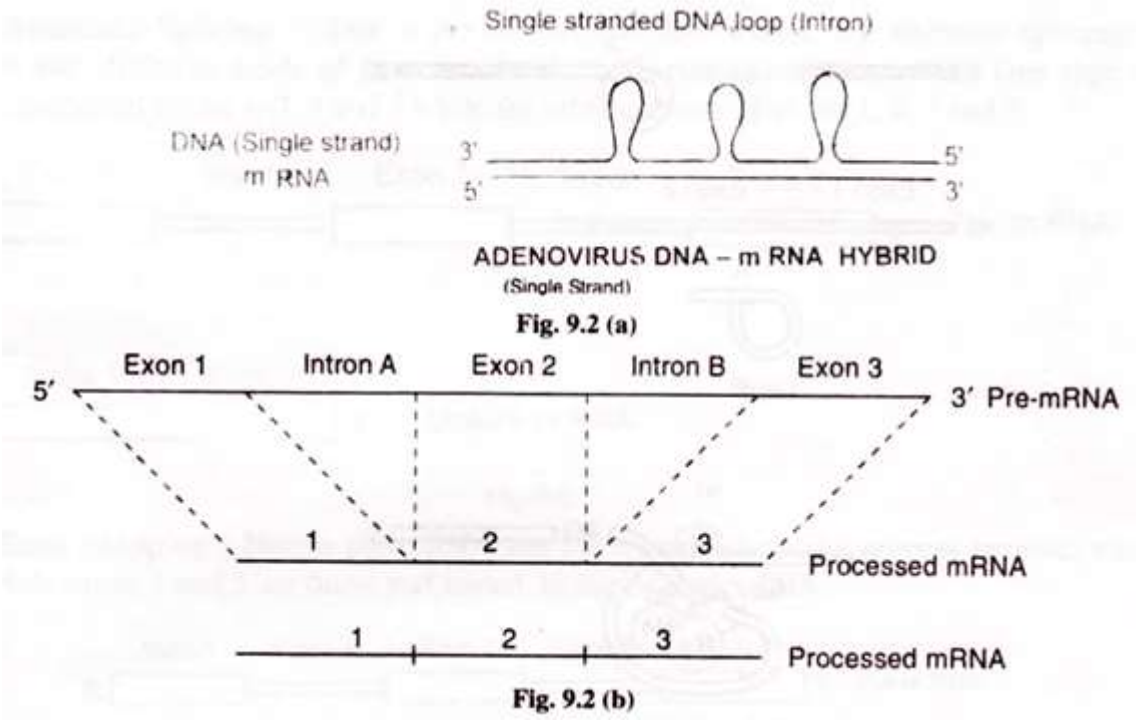


Fig. 9.2 (a)

Fig. 9.2 (b)

The splicing by excision is remarkably precise and accurate and cuts or nicks are made at highly precise position. Because even if an error of one base occurs, the correct reading frame would to disturbed and wrong amino acid will be coded. Chambon studied the boundaries of the introns.

The end of intron always have GU towards the 5′-end (5′-GU-3′) and AG towards the 3′-end (5′-AG-3′). This is known as GU-AG rule or chambon's rule according to which an intron begins with GU and ends with AG. The boundary between exon-intron at 5′-end is called 5′-splice site, while boundary between intron-exon at 3′-end is called 3′-splice site.

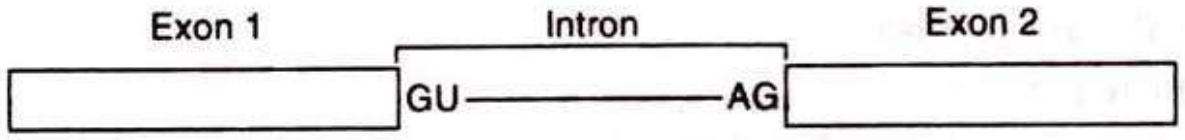


Fig. 9.3.

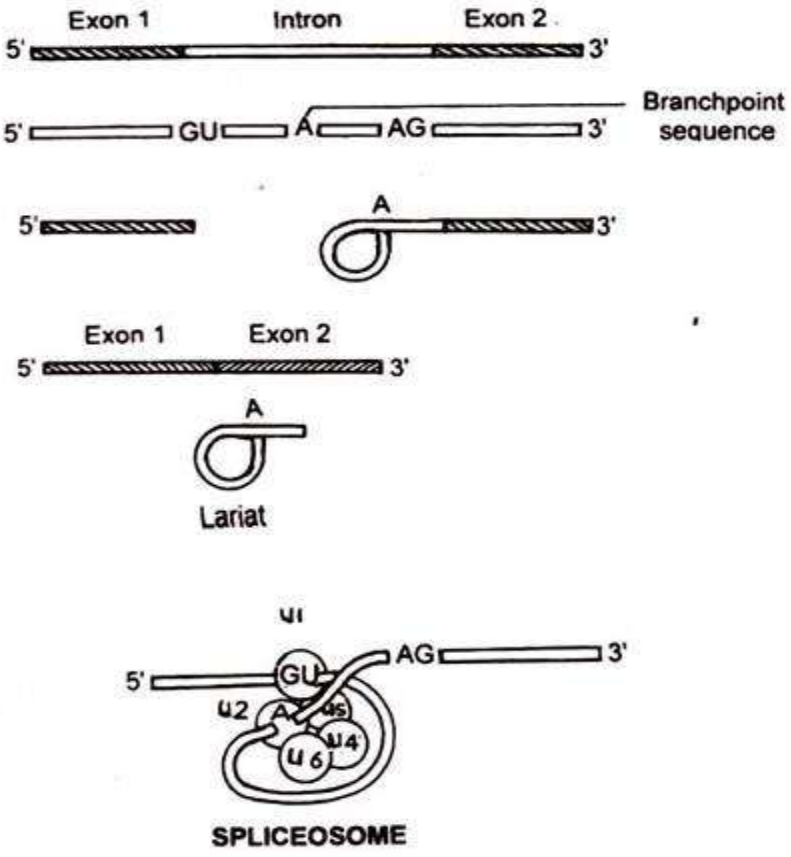
Introns are excised one by one. The AG at the 3′-end is preceded by a pyrimidine rich sequence called polypyrimidine tract (Py tract). About 10-40 bases upstream of the polypyrimidine tract is a sequence called branchpoint sequence (A), which is 5′-UACUAAC- 3′ in yeast. This sequence is required in lariat formation during splicing process.

RNA Splicing:

Splicing is generally performed by endonuclease enzymes cleaving the introns at both ends. Phosphodiester bond between sugar and phosphate at the junction between intron and exon is cleaved. The freed 5′-end of the intron joins the branch point sequence of form lariat.

Spliceosome:

Splicing is performed by a large complex called spliceosome. The spliceosome is made up of small nuclear ribonuclear proteins (sn RNP) called snurps. These consist of RNAs which are rich in uracil and are of several types U1, U2, U4, U5 and U6 which are collectively called small nuclear RNAs (sn RNA).



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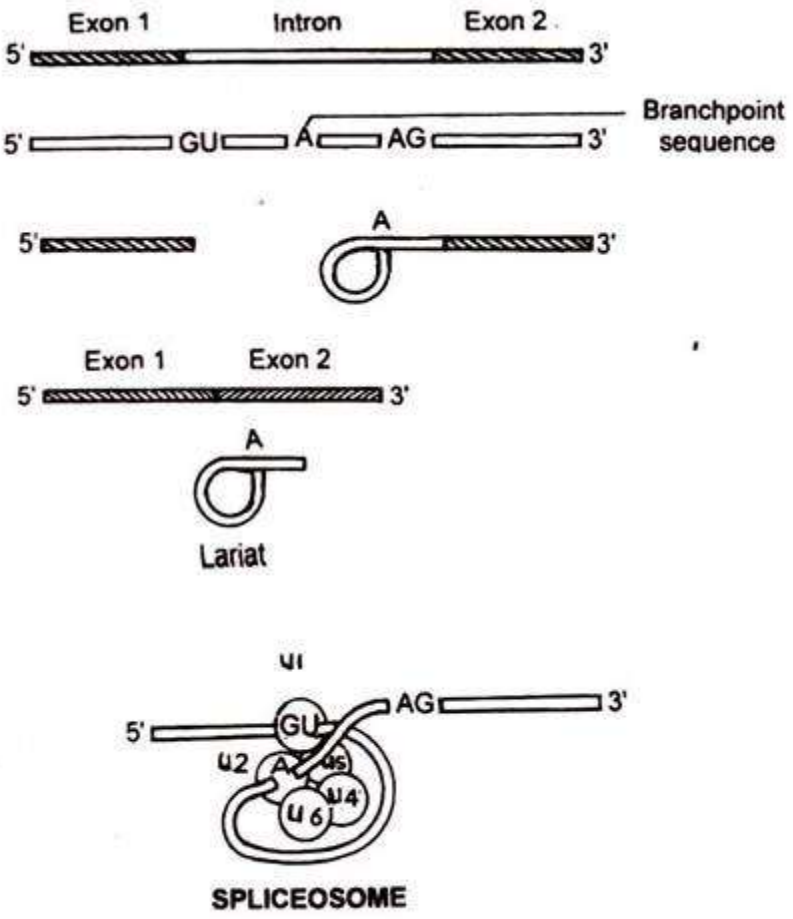


Fig. 9.4.

Mechanism of Splicing:

At first U1 snRNP recognize and-break the 5'splice site of the intron and bring it closer to branch site. Then the complex of sn RNP of U2, U4, U5 and U6 bind to the intron. The complex of snRNPs and precursor mRNA of the intron is called spliceosome. The spliceosome is looped out. This loop of intron is called lariat which is discarded and degraded. The exons on either side of the removed intron are brought closer and ligation seals them together.

Self Splicing:

In some cases specially group I introns, the intron itself folds and catalyses its own splicing. Here RNA of the intron functions as an enzyme and behaves like an endonuclease to splice out the intron. The RNA which acts as an enzyme is called ribozyme. There the splicing of the intron sequences requires no other enzyme. The intron is released in a linear form which is subsequently degraded.

Alternate Processing of Pre-mRNA:

Primary transcript (pre-mRNA) generally exhibits one pattern of RNA splicing to produce a single type of mature mRNA. But in higher eukaryotes, pre-mRNA exhibits alternate or differential processing patterns to yield different mRNAs containing different exons which encode different proteins. One pre-mRNA after alternate splicing produces more than one type of mature mRNAs.

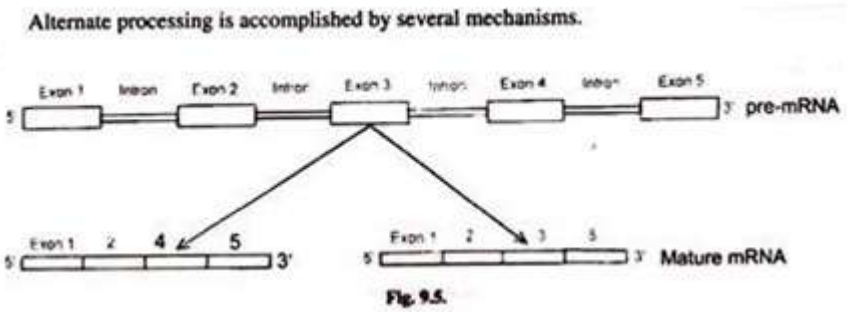


Fig. 9.5.

1. Alternate Splicing:

Here a pre-mRNA has five exons. By alternate splicing it produces two different kinds of mature mRNAs both containing four exons. One type of mRNA consists of exons 1, 2, 4 and 5 while the other consists of exons 1, 2, 3 and 5.

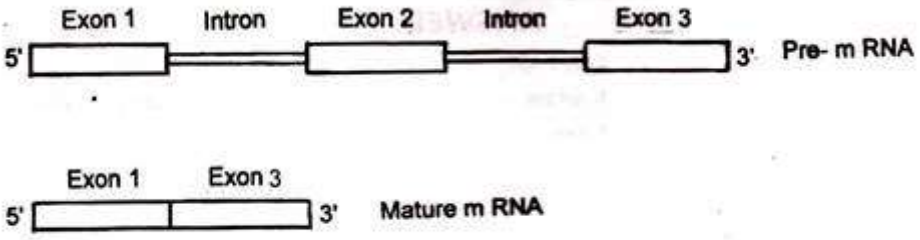


Fig. 9.6.

2. Exon Skipping:

Here a pre-mRNA has three exons. Splicing process removes exon no. 2, while exons 1 and 3 are fused and sealed, in the mature mRNA.

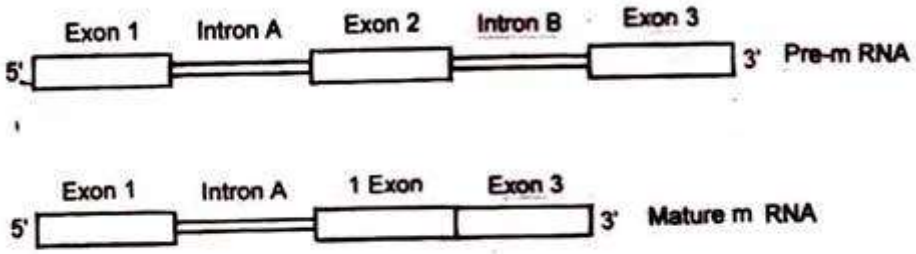


Fig. 9.7.

3. Intron Retaining:

Here a pre-mRNA has three exons and two introns. Splicing process removes the intron no. B while intron no. A is retained in the mature mRNA. It is possible to retain only a part of an intron.

D. RNA Editing:

Pre-mRNA may also undergo RNA editing in which the sequence of the pre- mRNA is altered by the insertion, deletion or substitution of bases. RNA editing was first identified in the mitochondrial gene in which the transcripts were found to be extensively modified by the insertion of uracil residues. RNA editing, may involve either the whole gene, i.e., pan-editing or just a few bases, i.e., minor editing.

Different types of RNA editing are:

(a) Base insertion/deletion type (Fig. 16.9C)

(i) U-insertion/deletion editing in the kinetoplastid, mitochondria is catalysed by editosome through cleavage, addition or removal and ligation utilizing guide RNAs (gRNAs).

(ii) C-insertion/dinucleotide (GC, GU, CU, AA, AU) editing in mitochondria of slime moulds is achieved by slippery transcription.

(iii) G or A-insertion editing found in negative strand RNA viruses and Ebola virus respectively.

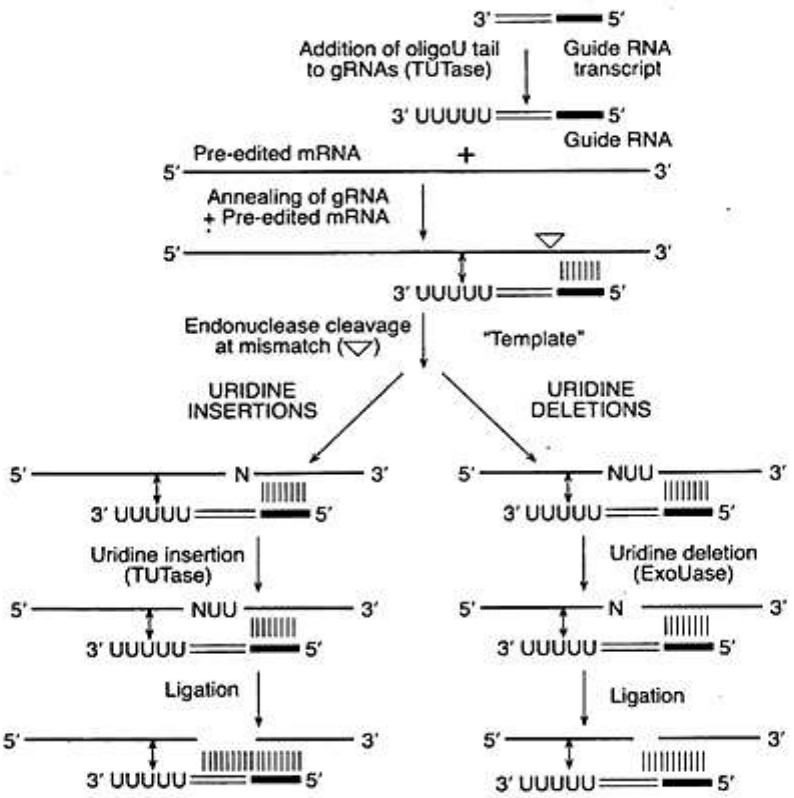


Fig. 16.9C: Mechanism of U insertion and deletion editing in trypanosome mitochondria. Enzymes that carry out each of the steps in U insertion and U deletion editing are shown. The site of pre-edited mRNA cleavage upstream of the anchor duplex is shown by a triangle. Guide RNA (gRNA) domains are indicated, with the 'anchor' as solid black segment, the 'guiding region' as double lined segment, and the 'oligo (U) tail' as UUUUU (from P. K. Gupta)

(b) Base substitution/modification type (Fig. 16.9D)

(i) C to U editing in plant mitochondria and chloroplasts, mammalian apo B, etc. involves transition due to deamination of cytosine by cytidine deaminase.
(ii) A to I (inosine) editing in glutamate receptor, hepatitis delta virus, etc. occurs through deamination of adenosine with the help of adenosine deaminases acting on RNA (ADARs) by specifically target- ting single nucleotides within partially double stranded pre-mRNAs.
(iii) A to G or U to A or U to G editing found in vertebrate mRNAs.

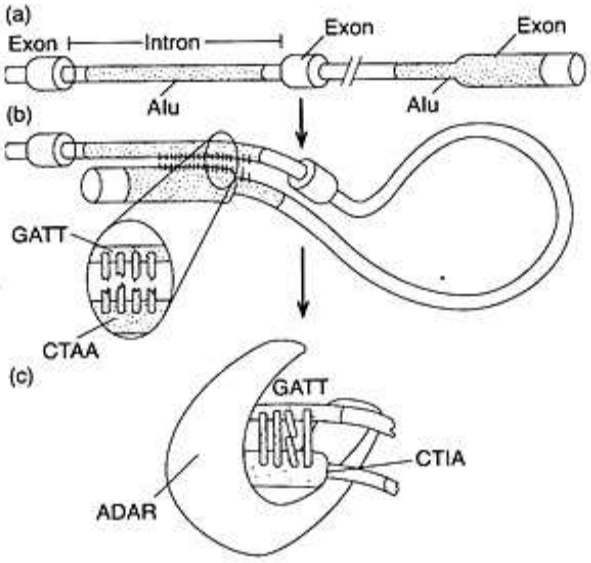


Fig. 16.9D: ADAR-mediated editing. (a) Pre-mRNA is transcribed from DNA. The gene contains two Alu repeats with opposite orientations, one of which overlaps with an exon. (b) The two oppositely oriented Alu sequnces form a dsRNA structure. (c) An enzyme of the ADAR family edits some of the adenosines (A) in the dsRNA structure into inosines (I) (from P. K. Gupta)

Processing of tRNA:

tRNA undergoes extensive processing. The mature tRNAs consists of 80-90 nucleotides. But the precursor tRNA is much longer. For example $tRNA^{Tyr}$ which is a tyrosine carrying tRNA contains 350 nucleotides. Processing discards useless sequences. This is done by enzymes RNase D, RNase E, RNase F and RNase P. Nucleotides are removed from both 5′ and 3′ ends. Endonucleases also remove many sequences. Cleaving is done after the primary transcript has folded and formed characteristic stems and loop structure by extensive complementary base pairing. RNase P is a ribozyme. The 5′-CCA-3′ sequence at 3′-end of mature tRNA is added by the enzyme tRNA nucleotidyl transferase. This generates the mature 3′-end the tRNA.

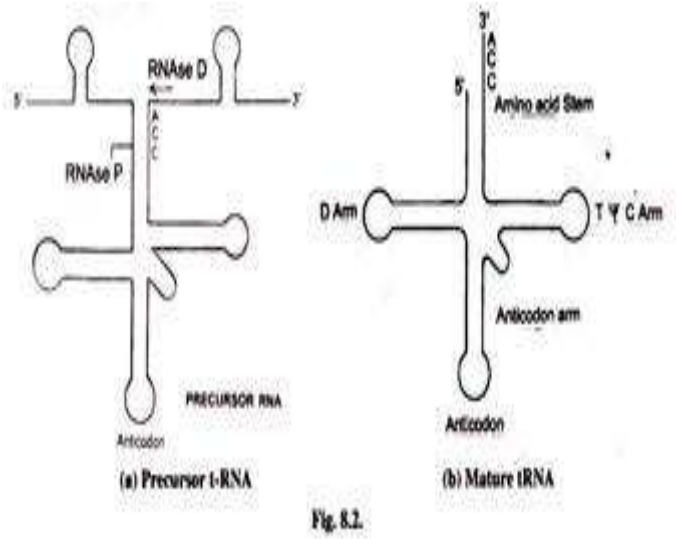


Fig. 8.2.

Several unusual bases are formed by the modification of normal existing bases A, G, C and U by the enzymatic action. These modified bases are pseudouridine (Ψ), 2- isopentenyladenosine (2 ip A), 2-O-methylguanosine (2m G), 4-thiouridine (4 t μ), Ribothymidine, dihyrouridine and inosine.

rRNA Processing:

In rRNA processing in both prokaryotes and eukaryotes, the primary transcript undergoes some changes. Some nucleotides are removed by exonucleases and endonucleases. New nucleotides are added both at 5′-end and 3′-end. Certain nucleotides are modified.

rRNA Processing in Prokaryotes:

In E. coli there are several different operons in rRNA. Each operon contains one copy of 5S, 16S and 23S rRNA sequences. In addition, several coding sequences of tRNA molecules are also present in rRNA operons. These primary transcriptions are processed to give both rRNA and tRNA molecules. All these molecules are cleaved from a continuous transcript of more than 5000 nucleotides.

rRNA Processing in Eukaryotes:

The precursor rRNA in eukaryotes contain one copy of 18S coding region, one copy of 5.8S and one copy of 28S coding region. This rRNA precursor is transcribed in the nucleolus by RNA polymerase I. The eukaryotic 5S rRNA is transcribed by RNA polymerase III from an unlinked gene. In both prokaryotes and eukaryotes rRNA molecules form secondary structures of numerous double stranded stems by complementary base pairing and single stranded loops.

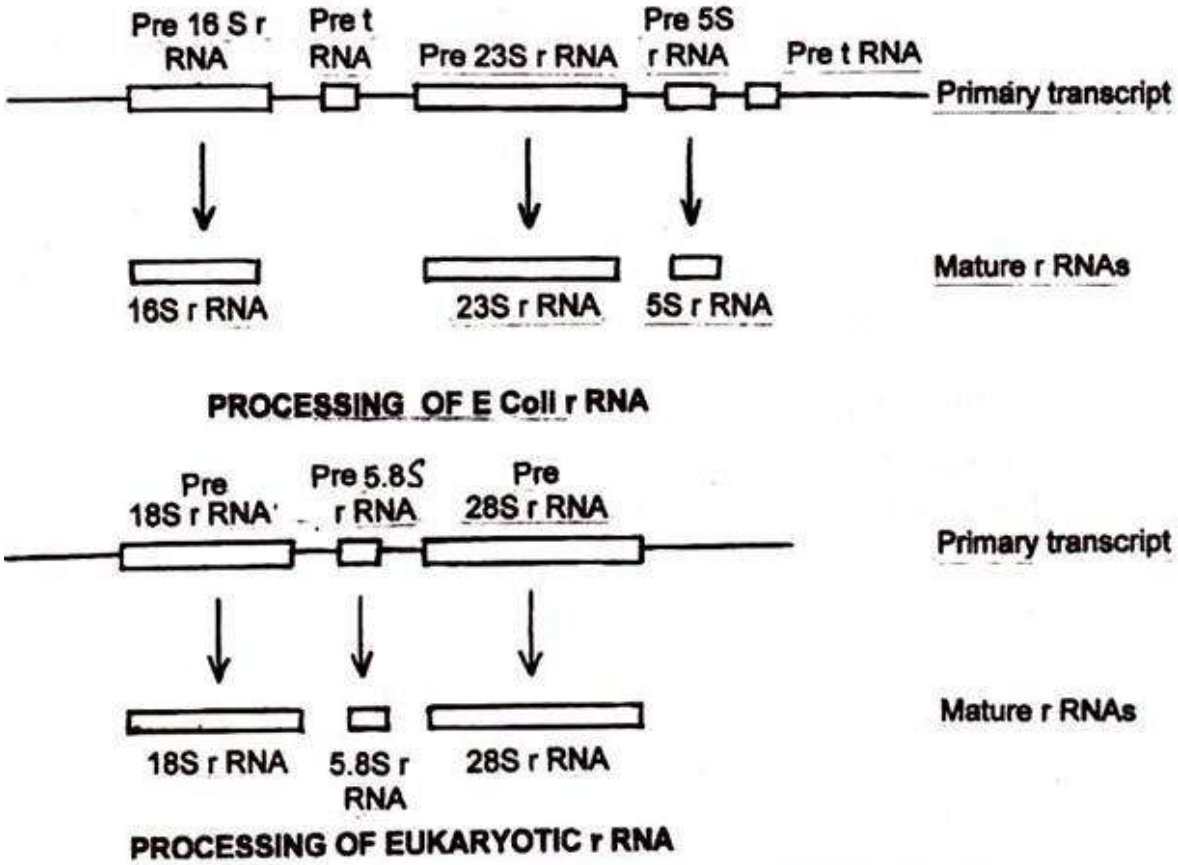


Fig. 8.3. Processing of rRNA primary transcript

Small nucleolar RNA (snoRNA) is required for the processing of rRNA molecules in eukaryotes. The snoRNAs are present in the nucleolus where rRNA is processed. Ribosomes are also assembled in nucleolus.

The RNA molecules in cells are present in the form of complexes formed with proteins. Specific proteins bind to specific RNAs. The RNA-protein complexes are called ribonuclear proteins or RNPs. Ribosomes are the largest RNPs formed by rRNA molecules forming complexes with specific ribosomal proteins. In E. coli ribosomes account for 25% of the dry weight of the cell. It consists of 10% of total proteins and 80% of total RNA. In prokaryotes 70S ribosome has 30S and 50S subunits. The 30S subunit has one copy of 16S rRNA and 21 different proteins. The 50S subunit has 23S rRNA, 5S rRNA and 31 different proteins.

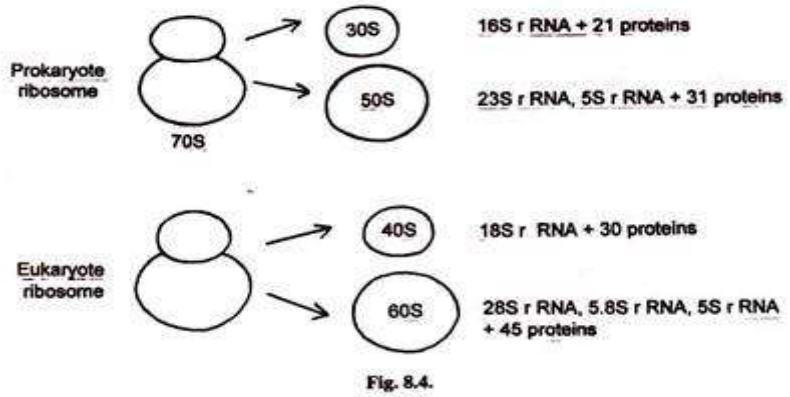


Fig. 8.4.

In eukaryotes 80S ribosome has 40S and 60S subunits. The 40S subunit has 18S rRNA and 30 different proteins. The 60S subunit has 28S rRNA, 5.8S rRNA, 5.8S rRNA and 45 different proteins.

Catalytic RNA:

Like protein enzymes some RNA molecules function as enzymes. Like protein enzymes some RNA sequences form complex tertiary structures and play the role of biological catalysts. Such RNA enzymes are known as ribozymes. Like enzymes they have an active site, a binding site for a substrate and a binding site for a co-factor such as metal ion.
Ribozymes have been extensively studied in rRNA of a ciliate protozoan *Tetrahymena thermophila* and are also found in fungal mitochondria, in phage T_4 and E. coli bacteria.

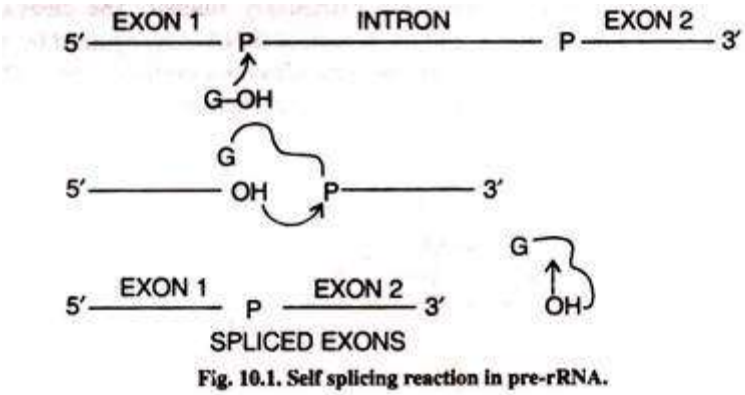


Fig. 10.1. Self splicing reaction in pre-rRNA.

Group 1 introns have an intrinsic ability to splice themselves which is called self splicing or autosplicing. Here no enzyme is required and RNA sequence functions as its own ribozyme and behaves like an endonuclease. In rRNA of Tetrahymena the splicing process involves binding of a single guanosine (G) to a site within the intron which is to be spliced out.
The splicing involves two transesterification reactions. First a free guanosine nucleotide attacks the phosphodiester bond at the 5′ splice site of the intron cleaving the 5′ end of intron. Second trans-esterification reaction splices the phosphodiester bond at the 3′-splice site of next exon causing cleavage. This causes the 3′ end of exon 1 to form a phosphodiester bond with 5′ end of exon 2, thus joining them. The intron is released in a linear form, which is subsequently degraded. It this way the RNA molecule functions as its own ribozyme as it splices itself without the help of any protein enzyme.
Another ribozyme is ribonuclease P (RNAse P) which is a ribonuclease involved in the processing of pre-tRNA. The RNAse P is an ribozyme composed of one RNA component and one protein component. Its RNA component is a ribozyme which catalyses the processing of precursors tRNA.

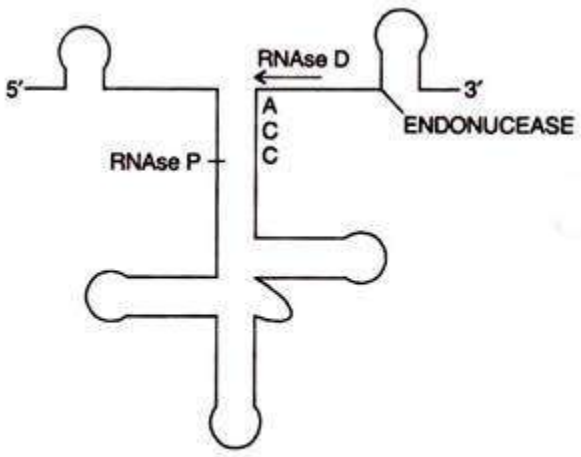


Fig. 2

Fig. 10.2. Role of ribozymes in processing of pre t-RNA.

Precursor tRNA is a very long molecule. During processing its large portion is removed and degraded. Precursor tRNA is cleaved at both 5′-end and 3′-end. In E. coli the tRNA which carries amino acid tyrosine ($tRNA^{lyr}$) has been extensively studied. The cleavage occurs differently at 5′-end and 3′-end. At the 5′-end the precursor tRNA is recognized by an enzyme known as RNAse P which is a ribozyme. This ribozyme cleaves a portion of pre-tRNA so that correct 5′-end of mature tRNA is produced. This proves that all enzymes are not proteins.

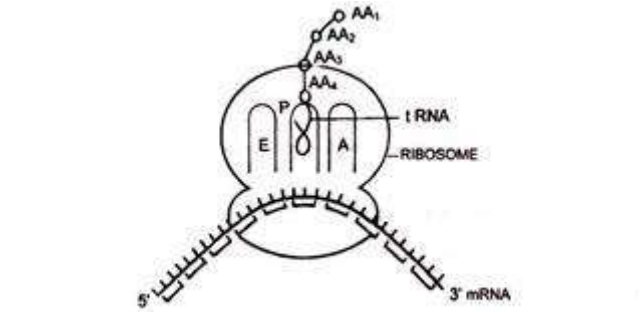


Fig. 10.3. 23S rRNA is a ribozyme responsible for catalyzing peptide bond formation between successive amino acids.

Role of Ribozyme in Protein Synthesis:

In protein synthesis, during elongation phase, the amino acids are added one-by-one. During elongation phase, a tRNA brings a new amino acid to the ribosome according to the codons of mRNA. Each new amino acid is then attached to the end of the growing polypeptide chain. During chain elongation, a peptide bond is formed between the amino acid at "A" site and growing polypeptide chain, which is transferred from "P" site to "A" site. The transfer is known as peptidyl transfer reaction.

This transfer involves formation of peptide bond between amino acid at the "A" site and polypeptide chain, thus lengthening the chain by one amino acid. It was discovered that the peptidyl transferase. Which catalyses the peptide bond formation between successive amino acids consists of several proteins and a $_{23}$S rRNA molecule present in the ribosome. This 23S rRNA is a ribozyme and is responsible for catalysing peptide bond formation between successive amino acid.

Hammerhead Ribozyme:

Hammerhead ribozyme is another small RNA which performs self cleavage reaction. It is found in viroids, which have RNA as genetic material and they infect the plants. When viroid replicates by rolling circle, it produces a continuous RNA chain, which consists of multiple copies of RNA. This continuous chain RNA undergoes cleavage to form single viroids or monomers. The self-cleaving RNA sequence at the junction of monomers is called hammerhead because of the shape of its secondary structure.

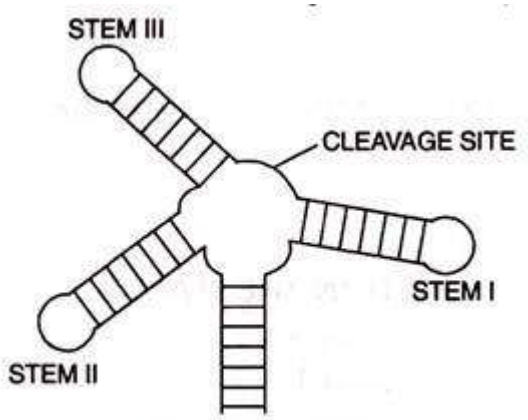


Fig. 10.4. Hammerhead Ribozyme

It consists of three complementary base paired stems. These are stem I, stem II and stem III surrounding a core of non-complementary nucleotides. This core lies at their junction and undergoes self-cleavage reaction. The catalytic reaction centre lies between stem 2/3 and stem I. This centre contains a magnesium ion that initiates hydrolysis reaction. The reaction breaks the RNA chain producing a 2′, 3′ cyclic phosphate and a free 5′-hydroxyl end. Like antisense RNA, which is used to silence the genes, rihozyme is also used to turn off specific genes.

Discovery of Split Genes:

During 1970, in some mammalian viruses (e.g. adenoviruses) it was found that the DNA sequences coding for a polypeptide were not present continuously but were split into several pieces.

Therefore, these genes were variously named as split genes or introns, interrupted genes or intervening sequences, inserts, Junk DNA. For the discovery of split genes in adenoviruses and higher organisms, Richards J. Roberts and Phillip Sharp were awarded Nobel Prize in 1993.

As shown in Fig 6.4 a DNA sequence codes for mRNA but the complete corresponding sequence of DNA is not found in mRNA. Certain sequences of DNA are missing in mRNA. The sequences present in DNA but missing in mRNA are called intervening sequences or introns, and the sequences of DNA found in RNA are known as exons. The exons code for mRNA. For the first time W. Gilbert used the term introns and exons. After transcription a limited RNA transcript has the intron. Genes coding for rRNA and tRNA may also be intervened. The introns are also found in some eubacteria, cyanobacteria and archaeobacteria. For some time it was not certain how mRNA is synthesized from a DNA containing introns.

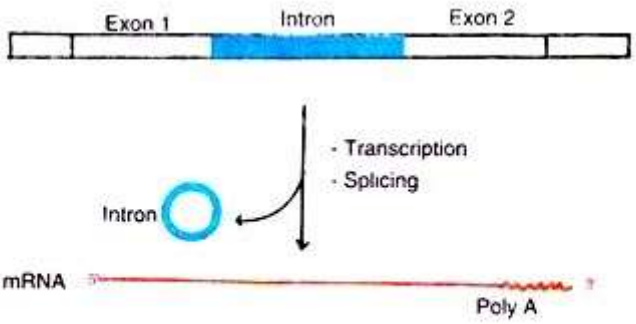


Fig. 6.4 : The split genes have exons separated by introns. Removal of introns through RNA splicing.

Evolution of Split Genes*:*

Before the discovery of split genes in 1977, all the genes analysed in detail were the bacterial genes. Bacteria were considered to resemble with the simpler cell from which eukaryotes must have been evolved. Now, it is supposed that split genes are the ancient condition and bacteria lost their introns only after evolution of most of their proteins.

Evidence for the ancient origin of introns has been obtained by the examination of the gene that encodes the ubiquitous enzyme, triose phosphate isomerase (TPI). The TPI is coded by a gene that contains six introns (in vertebrates), five of these are present at the same position as in maize. This shows that five introns were present in the gene before evolution of eukaryotes about 10^9 years ago.

The TPI plays a key role in cell metabolism that catalyses the inter-conversion of glyceraldehyde 3-phosphate and dihydroxy acetone phosphate- a central step in glycolysis and glycogenesis. By comparing this enzyme in various organisms it appears that the TPI evolved before the divergence of prokaryotes and eukaryotes from a common ancestor cell progenote.

The unicellular organisms under a strong selection pressure minimised the superfluous genome in their cell, whereas there was no such pressure on multicellular organisms. That is why Aspergillus has five introns and Saccharomyces has none. Precise loss of introns would have occurred by deletion in prokaryotes. The loss of introns requires the exact rejoining of DNA coding sequence.

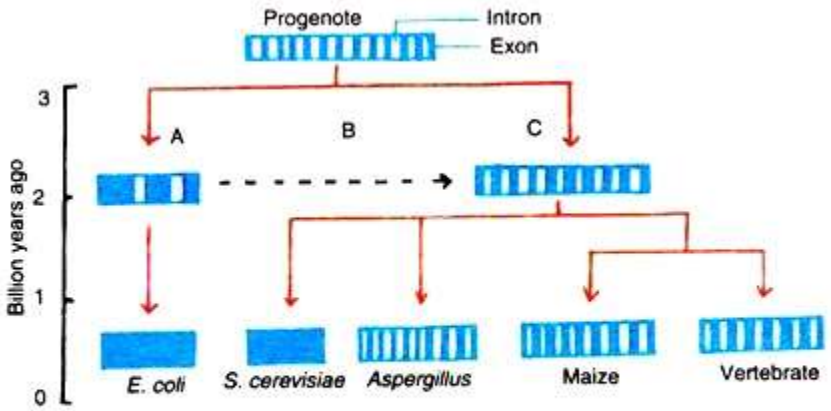


Fig. 6.5 : Outline of evolution of a particular gene. Introns were present in progenote before the evolution of archaeobacteria, eubacteria and eukaryotes. A, eubacteria that formed mitochondria and chloroplasts; B, approximate time of the endosymbiotic events that gave to mitochondria and chloroplast, and C, anaerobic eukaryotes.

The most likely source of the information is needed for such event in an mRNA transcript of the original gene from which introns are to be removed. The mRNA may be copied back into DNA by reverse transcriptase.
The recombination enzymes allow the DNA copies to become paired with the original sequence resulting in intron less form by a gene-conversion type of event. This pathway of intronless has been demonstrated in laboratory in S. cerevisiae.

Alternative splicing:

Alternative splicing, or differential splicing, is a regulated process during gene expression that results in a single gene coding for multiple proteins. In this process, particular exons of a gene may be included within or excluded from the final, processed messenger RNA (mRNA) produced from that gene. Consequently, the proteins translated from alternatively spliced mRNAs will contain differences in their amino acid sequence and, often, in their biological functions. Notably, alternative splicing allows the human genome to direct the synthesis of many more proteins than would be expected from its 20,000 protein-coding genes.

Alternative splicing occurs as a normal phenomenon in eukaryotes, where it greatly increases the biodiversity of proteins that can be encoded by the genome; in humans, ~95% of multi-exonic genes are alternatively spliced. There are numerous modes of alternative splicing observed, of which the most common is exon skipping. In this mode, a particular exon may be included in mRNAs under some conditions or in particular tissues, and omitted from the mRNA in others.

The production of alternatively spliced mRNAs is regulated by a system of trans-acting proteins that bind to cis-acting sites on the primary transcript itself. Such proteins include splicing activators that promote the usage of a particular splice site, and splicing repressors that reduce the usage of a particular site. Mechanisms of alternative splicing are highly variable, and new examples are constantly being found, particularly through the use of high-throughput techniques. Researchers hope to fully elucidate the regulatory systems involved in splicing, so that alternative splicing products from a given gene under particular conditions ("splicing variants") could be predicted by a "splicing code". Abnormal variations in splicing are also implicated in disease; a large proportion of human genetic disorders result from splicing variants. Abnormal splicing variants are also thought to contribute to the development of cancer, and splicing factor genes are frequently mutated in different types of cancer.

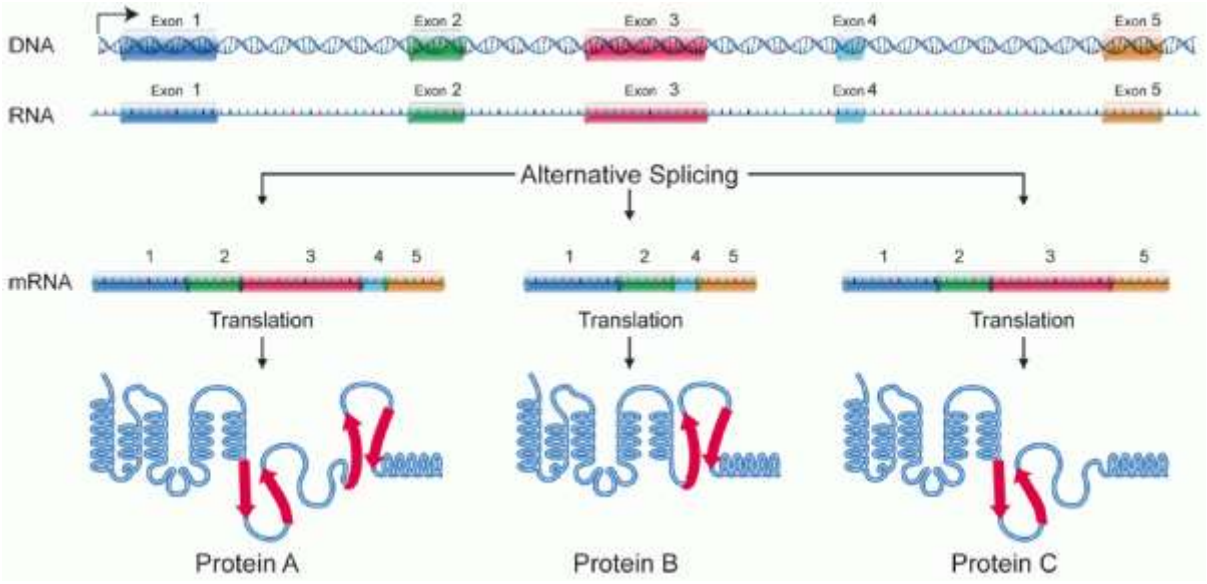


Figure: Alternative splicing produces three protein isoforms.

Five basic modes of alternative splicing are generally recognized.

- Exon skipping or cassette exon: in this case, an exon may be spliced out of the primary transcript or retained. This is the most common mode in mammalian pre-mRNAs.
- Mutually exclusive exons: One of two exons is retained in mRNAs after splicing, but not both.
- Alternative donor site: An alternative 5' splice junction (donor site) is used, changing the 3' boundary of the upstream exon.
- Alternative acceptor site: An alternative 3' splice junction (acceptor site) is used, changing the 5' boundary of the downstream exon.
- Intron retention: A sequence may be spliced out as an intron or simply retained. This is distinguished from exon skipping because the retained sequence is not flanked by introns. If the retained intron is in the coding region, the intron must encode amino acids in frame with the neighbouring exons, or a stop codon or a shift in the reading frame will cause the protein to be non-functional. This is the rarest mode in mammals.

In addition to these primary modes of alternative splicing, there are two other main mechanisms by which different mRNAs may be generated from the same gene; multiple promoters and multiple polyadenylation sites. Use of multiple promoters is properly described as a transcriptional regulation mechanism rather than alternative splicing; by starting transcription at different points, transcripts with different 5'-most exons can be generated. At the other end, multiple polyadenylation sites provide different 3' end points for the transcript. Both of these mechanisms are found in combination with alternative splicing and provide additional variety in mRNAs derived from a gene.

Adaptive significance:

Alternative splicing is one of several exceptions to the original idea that one DNA sequence codes for one polypeptide (the One gene-one enzyme hypothesis). It might be more correct now to say "One gene – many polypeptides". External information is needed in order to decide which polypeptide is produced, given a DNA sequence and pre-mRNA. Since the methods of regulation are inherited, this provides novel ways for mutations to affect gene expression.

It has been proposed that for eukaryotes alternative splicing was a very important step towards higher efficiency, because information can be stored much more economically. Several proteins can be encoded by a single gene, rather than requiring a separate gene for each, and thus allowing a more varied proteome from a genome of limited size. It also provides evolutionary flexibility. A single point mutation may cause a given exon to be occasionally excluded or included from a transcript during splicing, allowing production of a new protein isoform without loss of the original protein. Studies have identified intrinsically disordered regions as enriched in the non-constitutive exons[35] suggesting that protein isoforms may display functional diversity due to the alteration of functional modules within these regions. Such functional diversity achieved by isoforms is reflected by their expression patterns and can be predicted by machine learning approaches. Comparative studies indicate that alternative splicing preceded multicellularity in evolution, and suggest that this mechanism might have been co-opted to assist in the development of multicellular organisms.

Research based on the Human Genome Project and other genome sequencing has shown that humans have only about 30% more genes than the roundworm *Caenorhabditis elegans*, and only about twice as many as the fly *Drosophila melanogaster*. This finding led to speculation that the perceived greater complexity of humans, or vertebrates generally, might be due to higher rates of alternative splicing in humans than are found in invertebrates. However, a study on samples of 100,000 ESTs each from human, mouse, rat, cow, fly (*D. melanogaster*), worm (*C. elegans*), and the plant *Arabidopsis thaliana* found no large differences in frequency of alternatively spliced genes among humans and any of the other animals tested.[41] Another study, however, proposed that these results were an artefact of the different numbers of ESTs available for the various organisms. When they compared alternative splicing frequencies in random subsets of genes from each organism, the authors concluded that vertebrates do have higher rates of alternative splicing than invertebrates.

Micro RNA and other non coding RNA:

Micro RNAs were first described for the worm C. elegans in 1993 by Lee and colleagues in the Victor Ambros lab. However, the term micro RNA was only introduced in 2001. By 2002, miRNAs have been confirmed in various plants and animals, including C. elegans, human and the plant *Arabidopsis thaliana*. Work at the University of Louisville has resulted in the production of microarrays containing all known miRNAs for human, mouse, rat, dog, C. elegans and Drosophila.
In genetics, micro RNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. In other words, a non-coding RNA molecule of approximately 21-23 nucleotides that inhibits mRNA expression is known as micro RNA.

The main points about micro RNA are given below:

1. Micro RNA is involved in regulation of gene expression.
2. In a cell, miRNA is transcribed from DNA but not translated into proteins.
3. Micro RNAs are non-coding molecules of approximately 21-23 nucleotides.
4. Micro RNAs inhibit the expression of mRNA molecule.
5. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules.
6. The main function of miRNAs is to down regulate gene expression.
7. It has been reported that a typical mammalian cell contains as many as 50,000 different miRNAs.
8. Micro RNAs were first described nor the worm *C. elegans* in 1993.
9. The term micro RNA was only introduced in 2001.
10. Only one strand of DNA can function as templates to give rise to miRNA.

Formation of Micro RNAs (miRNA):

The formation of micro RNAs consists of three important steps, viz:
(i) Formation of primary miRNA,
(ii) Formation of precursor miRNA, and
(iii) Formation of mature functional miRNA.

These are discussed below:

1. Formation of Primary miRNA:

The primary transcript is synthesized from DNA template. The miRNAs are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and then processed to pre-miRNA. Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

2. Formation of Precursor miRNA from pre-miRNA:

A short, 70-nucleotide stem-loop structure known as pre-miRNA is formed from primary miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha.

3. Formation of Mature miRNA from pre-miRNA:

These pre-miRNAs are then processed to mature miRNAs in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC). This complex is responsible for the gene silencing observed due to miRNA expression and RNA interference. The genes encoding miRNAs are much longer than the processed mature miRNA molecule.

Thus microRNA (miRNA) is produced from precursor microRNA (pre-miRNA), which is formed from a microRNA primary transcript (pri-miRNA). The process of formation of miRNA can be represented as follows.

DNA ⟶ Primary transcript (pri-miRNA) ⟶ Precursor miRNA (pre-miRNA)
⟶ mature functional miRNA

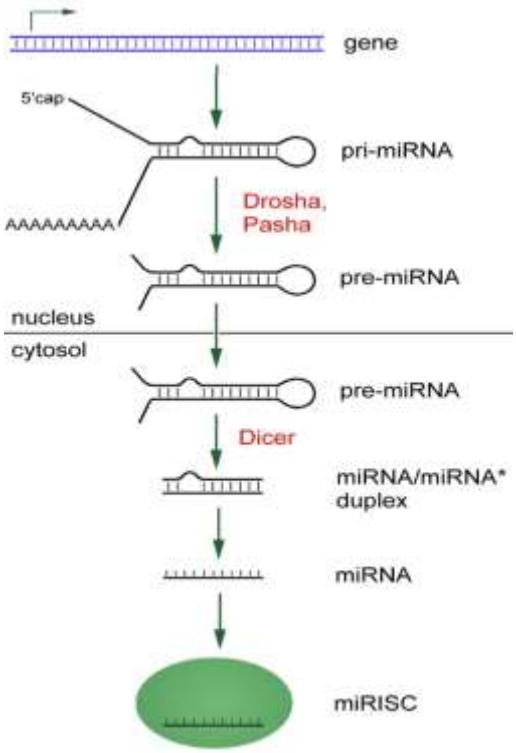


Figure: Different steps of Micro RNA formation

Processing of Micro RNAs (miRNA):

Efficient processing of pre-miRNA by Drosha requires the presence of extended single-stranded RNA on both 3′- and 5′-ends of hairpin molecule. These ssRNA motifs could be' of different composition but their length is important for processing. A bioinformatics analysis of human and fly pri-miRNAs revealed very similar structural regions, called 'basal segments', 'lower stems', 'upper stems' and 'terminal loops'.

Based on these conserved structures, thermodynamic profiles of primiRNA have been determined. The Drosha complex cleaves RNA molecule -2 helical turns away from the terminal loop and ~1 turn away from basal segments. In most analysed molecules this region contains unpaired nucleotides and the free energy of the duplex is relatively high compared to lower and upper stem regions.

Most pre- miRNAs do not have a perfect double-stranded RNA (dsRNA) structure topped by a terminal loop. Clear similarities between pri-miRNAs encoded in respective (5′- or 3′-) strands have been demonstrated. The pre-miRNA stem-loop is cleaved by Dicer into two complementary short RNA molecules, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein. The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate. After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce mRNA degradation by argonaute proteins, which are catalytically active members of the RISC complex.

Differences Between Small Interfering RNA (siRNA) and Micro RNA (miRNA):

Micro RNA [miRNA] is a short (about 21 to 23 nucleotides) single-stranded RNA molecule that is now recognized as playing an important role in gene regulation. It has some similarities and some differences with small interfering RNA (siRNA).

Both miRNA and siRNA have gene regulation functions, but there are slight differences. The miRNA may be slightly shorter [21-23 nucleotides] than siRNA (20 to 25 nucleotides). The miRNA is single-stranded, while siRNA is formed from two complementary strands. The two kinds of RNAs regulate genes in slightly different ways.

The miRNA attaches to a piece of messenger RNA (mRNA)—which is the master template for building a protein – in a non-coding part at one end of the molecule. This acts as a signal to prevent translation of the mRNA into a protein. siRNA, on the other hand, attaches to a coding region of mRNA, and so it physically blocks translation.

TABLE 20.1. Comparison of small interference RNA (siRNA) and microRNA (miRNA)

S.No.	*Particulars*	*siRNA*	*miRNA*
1.	Function	Gene regulation	Gene regulation
2.	Size in nucleotides	20-25	21-23
3.	Strands	Two complementary	Single
4.	Attachment to mRNA	Coding region	Non-Coding region
5.	Effect on translation	Translation is blocked	Translation is blocked

Roles of Micro RNA [miRNA]:

The miRNAs play important role in gene regulation. Micro RNAs arc also expected to be useful in detection of various diseases and their treatment in the years ahead.

The role of miRNAs in gene regulation and disease detection are briefly discussed as follows:

1. Gene Regulation:

The important cellular function of miRNAs is related to gene regulation. The miRNA is attached to the mRNA at a specific point and inhibits protein translation. In other words, the miRNA complex blocks the protein translation machinery. This is thought to be the primary mode of action of plant miRNAs.

In such cases, the formation of the double-stranded RNA through the binding of the miRNA leads to the degradation of the mRNA transcript. It is also believed' that miRNA can prevent translation without causing degradation of the mRNA.

2. Micro RNA and Diseases:

The discovery of miRNA has opened up new areas of research. Now miRNA-based diagnostics and therapeutics are getting increasing importance. The miRNA technology is expected to help in diagnosis and treatment of serious diseases like cancer, heart diseases and diseases related to the nervous system.

This will also help in reclassification of different types of cancers. Thus miRNA technology has wide applications in several areas such as cardiac research; virology, cell biology in general and plant biology.

Studies on miRNA expression profiling demonstrated that expression levels of specific miRNAs changed in diseased human hearts, pointing to their involvement in cardiomyopathies.

Furthermore, studies on specific miRNAs in animal models have identified distinct roles for miRNAs both during heart development and under pathological conditions, including the regulation of key factors important for cardio genesis, the hypertrophic growth response, and cardiac conductance. Similarly, several miRNAs have been found to have links with some types of cancer.

Concept of RNA Interference Technology

RNA (RNAi) interference is an exciting field in biotechnology. This is a new approach for achieving shut down of target gene. In RNA interference method only a few double-stranded RNA (dsRNA) molecule will be employed per cell to silence the expression of target gene.
Therefore, it is invaluable research tool for down regulation of gene expression particularly in mammalian cells. It is several thousand times powerful than antisense technology. RNA interference was first discovered in worms in 1998. RNA interference operates in plants and other organisms like fungi and mammals. It is also a powerful regulatory process in viral defence and for transposon silencing in plans and animals. Presently, this technique is widely used for medical purpose in silencing of disease related genes.
Intense study on molecular mechanism of RNAi showed that dsRNA molecules are triggered in the partial study of gene silencing. Like antisense oligonucleotides, SiRNA or RNAi can be produced by chemical synthesis and delivered exogenously to cells or SiRNA can be induced by transfection of plasmids that express SiRNA endogenously under the control of RNA pol III promoters. When double-stranded RNA is enter/available in cells, it is recognised and cut into pieces (21-23 bp) by an enzyme called Dicer. It is a member of RNase III family in ATP dependent progressive manner. These 21-23 bp long with short overhangs is known as short interference RNA or SiRNA. In the next step of duplex SiRNA are the unwind by a helicase activity assemble with special proteins to form what is known as RNA induced silencing complex (RISC).
This is otherwise called as sequence-specific mRNA eating machine. Unwinding of SiRNA takes place using ATP and one of the strands recognise complementary mRNA of target gene in the cell. Consequently double-stranded RNA is formed. This dsRNA from mRNA then becomes a substrate for Dicer cleavage activity, which results in the destruction of mRNA and formation of new SiRNA.

Degradation of RNA:

The concentration of an mRNA is a function of both its rate of synthesis and its rate of degradation. For this reason, if two genes are transcribed at the same rate, the steady-state concentration of the corresponding mRNA that is more stable will be higher than the concentration of the other. The stability of an mRNA also determines how rapidly synthesis of the encoded protein can be shut down. For a stable mRNA, synthesis of the encoded protein persists long after transcription of the gene is repressed. Most bacterial mRNAs are unstable, decaying exponentially with a typical half-life of a few minutes. For this reason, a bacterial cell can rapidly adjust the synthesis of proteins to accommodate changes in the cellular environment. Most cells in multicellular organisms, on the other hand, exist in a fairly constant environment and carry out a specific set of functions over periods of days to months or even the lifetime of the organism (nerve cells, for example). Accordingly, most mRNAs of higher

eukaryotes have half-lives of many hours. However, some proteins in eukaryotic cells are required only for short periods of time and must be expressed in bursts. For example, certain signaling molecules called cytokines which are involved in the immune response of mammals, are synthesized and secreted in short bursts. Similarly, many of the transcription factors that regulate the onset of the S phase of the cell cycle, such as c-Fos and c-Jun, are synthesized for brief periods only. Expression of such proteins occurs in short bursts because transcription of their genes can be rapidly turned on and off and their mRNAs have unusually short half-lives, on the order of 30minutes or less.

Cytoplasmic mRNAs are degraded by one of the pathways shown in Figure 12-29. For most mRNAs, the length of the poly(A) tail gradually decreases with time through the action of a deadenylating nuclease. When it is shortened sufficiently, PABPI molecules can no longer bind and stabilize interaction of the 5_ cap and initiation factors. The exposed cap then is removed by a decapping enzyme, and the unprotected mRNA is degraded by a 5' → 3'exonuclease. Removal of the poly(A) tail also makes mRNAs susceptible to degradation by cytoplasmic exosomes containing3' → 5' exonucleases. The 5'→ 3' exonucleases predominate in yeast, and the 3'→ 5' exosome apparently predominates in mammalian cells. For mRNAs degraded in these deadenylation-dependent pathways, the rate at which they are deadenylated controls the rate at which they are degraded. The rate of deadenylation varies inversely with the frequency of translation initiation for an mRNA: the higher the frequency of initiation, the slower the rate of deadenylation. This relation probably is due to the reciprocal interactions between initiation factors and PABPI that stabilize the binding of PABPI to the poly(A) tail, thereby protecting it from the deadenylation exonuclease. Many short-lived mRNAs in mammalian cells contain multiple, sometimes overlapping, copies of the sequence AUUUA in their 3' untranslated region. Specific RNA-binding proteins have been found to bind to these 3' AU-rich sequences. Recent experiments suggest that the bound proteins interact with a deadenylating enzyme and with the exosome, thereby promoting the rapid deadenylation and subsequent 3' → 5' degradation of these mRNAs. In this mechanism, the rate of mRNA degradation is uncoupled from the frequency of translation. Thus mRNAs containing the AUUUA sequence can be translated at high frequency, yet also degraded rapidly, allowing the encoded proteins to be expressed in short bursts. Some mRNAs are degraded in pathways that do not involve significant deadenylation. In one of these, mRNAs are decapped before the poly(A) tail is shortened extensively. It appears that certain mRNA sequences make the cap sensitive to the decapping enzyme, but the precise mechanism is unclear. In the other alternative pathway, mRNAs first are cleaved internally by endonucleases. The RNA-induced silencing complex (RISC) discussed earlier is an example of such an endonuclease. The fragments generated by internal cleavage then are degraded by exonucleases.

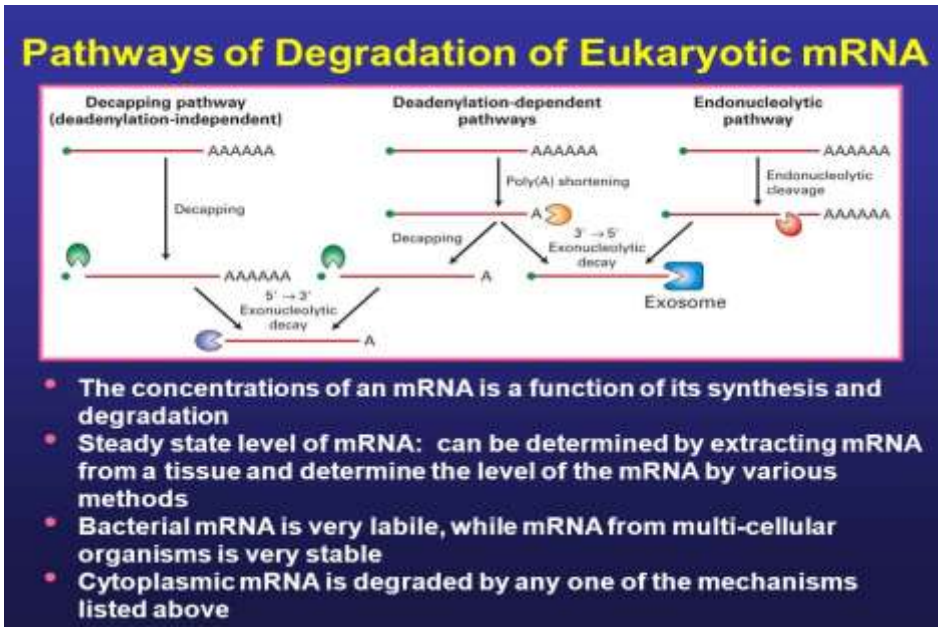


Fig: Pathways for degradation of eukaryotic mRNAs. In the deadenylation-dependent (*middle*) pathways, the poly(A) tail is progressively shortened by a deadenylase (orange)until it reaches a length of 20 or fewer A residues at which the interaction with PABPI is destabilized, leading to weakened interactions between the 5' cap and translation initiation factors. The deadenylated mRNA then may either (1) be decapped and degraded by a 5' → 3' exonuclease or (2) be degraded by a3' → 5' exonuclease in cytoplasmic exosomes. Some mRNAs(*right*) are cleaved internally by an endonuclease, and the fragments degraded by an exosome. Other mRNAs are decapped before they are deadenylated, and then degraded by a 5' → 3' exonuclease.

Probable Questions:

1. How 5’ capping is doe in eukaryotic mRNA?
2.How ply A tailing is done in eukaryotic mRNA?
3. What is intron ? How it is evolved?
3. What is spliceosome? How it removes introns from hnRNA ?
4. What is self splicing of RNA ? Describe the process.
5. What is exon skipping?
6. Describe different types of RNA editing.
7. How nascent rRNAs are processed?
8. How nascent tRNAs are processed?
9. What is ribozyme? How it is used in protein synthesis.
10. What is alternative splicing? How it enhances proteome diversity?
11. How mature miRNA are produced? Explain with diagram.
12. Compare siRNA and miRNA?
13. How RNAs are degraded? Describe in detail.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-III

Transport across the nuclear envelope - structure of nuclear membrane and nuclear pore complexes, processes of nuclear import and export and their regulation.

Translational machinery and translational control - energetics of amino acid polymerization, tRNAs and their modifications, aminoacyl tRNA synthetases, accuracy during aminoacylation

Objective: In this unit we will discuss nuclear membrane structure and export through nuclear pore. We will also discuss about translation process, tRNA structure and modification, RNA degradation and accuracy during aminoacylation.

Structure of Nuclear Membrane:

The nuclear membrane or karyotheca form an envelope-like structure around the nuclear contents and is commonly known as nuclear envelope. The nuclear membrane in higher plant and animal disappears in late prophase during mitosis and re-forms around the daughter chromosomes during telophase. In lower eukaryotes, the nuclear envelope remains intact throughout mitosis.

It separates nucleus from cytoplasm and functions to facilitate and regulate nucleocytoplasmic interaction. The light microscope provides little information about the nuclear envelope. Under electron microscope the nuclear envelope in the interphase or prophase stage appears to consist of two concentric membranes, viz., inner nuclear membrane and outer nuclear membrane.

Each membrane is about 75 to 90 A thick and lipoproteinous in nature. The outer and inner membranes are separated by perinuclear space of 100-170 A^0. The inter-membrane space is known as perinuclear cisternae . The inner membrane defines the content of nucleus itself and it contains specific proteins that act as binding sites for the nuclear lamina.

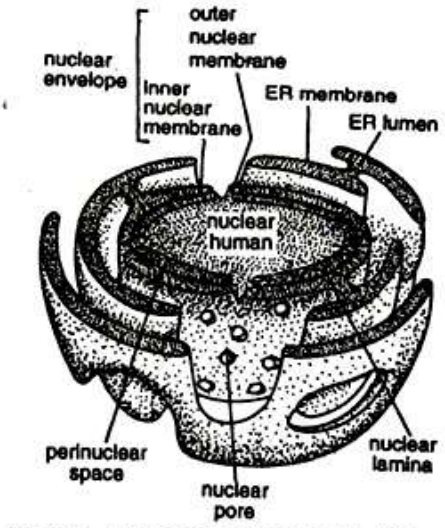


Fig. 9.4: A three-dimensional sketch of the double-membrane envelope that surrounds the nucleus. The nuclear envelope is penetrated by nuclear pores and is continuous with the endoplasmic reticulum.

The outer membrane is rough due to presence of ribosomes (25 nm in diameter) attached with it. The ribosomes are engaged in protein synthesis. The proteins made on these ribosomes are transported into the space between the inner and outer nuclear membrane.
In many cells, the outer nuclear membrane is continuous with rough endoplasmic reticulum. The space between the inner and outer nuclear membrane is continuous with the lumen or inner cavity of the rough endoplasmic reticulum.

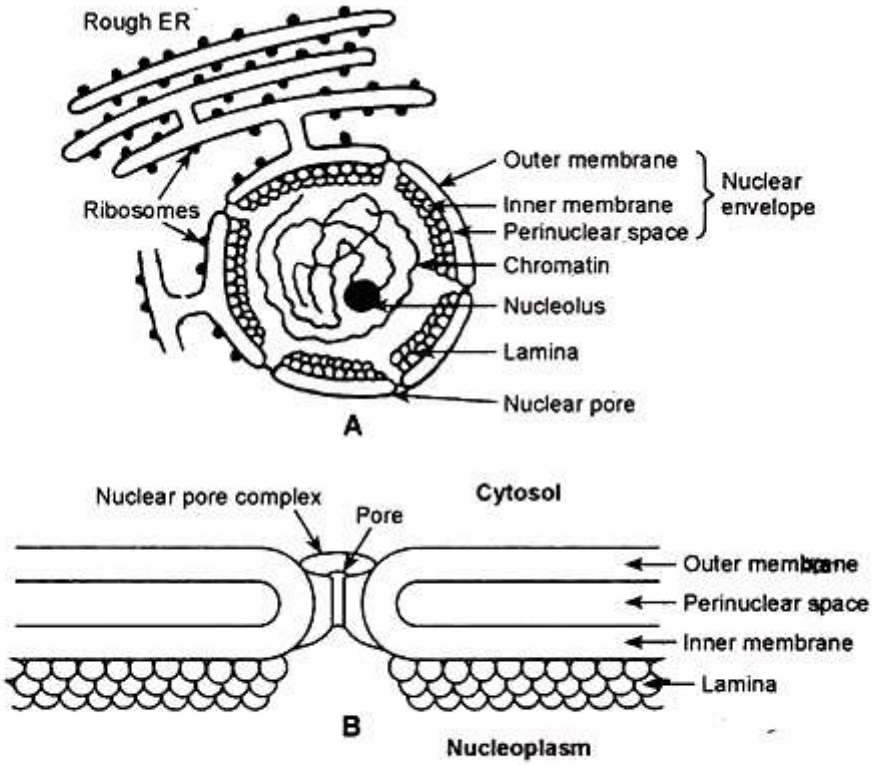


Fig. 2.22. Diagrammatic representation of a nucleus and a nuclear pore complex. **A.** Nuclear envelope connected with ER (out side) and associated with lamina on the inside. **B.** Part of nuclear envelope showing connection of outer and inner membrane at the pore. Lamina is adjacent to the nucleoplasm side of inner membrane.

Under the electron microscope the nuclear envelope appears to consist of two membranes, the outer and the inner nuclear membranes, separated by a perinuclear space of 20 nm (. Each of the two membranes of the nuclear envelope appears to have trilaminar unit membrane structure of 7 – 10 nm thickness. The outer nuclear membrane communicates with endoplasmic reticulum at several points and has ribosomes on the outer side.
The nuclear envelope is perforated by many circular apertures called nuclear pores. Each nuclear pore shows the presence of an electron- dense ring or cylinder called the annulus. The actual opening of the nuclear pore is thus confined to the cavity of the annulus. The annulus extends both into the cytoplasm and the nucleoplasm. The annulus typically consists of eight subunits arranged in radial symmetry around the periphery of the pore. The subunits have been variously interpreted as micro-cylinders, filaments, spheres or ovoid's.
A central ribonucleoprotein granule of 10-15 nm size may be present in some pore complexes and may be absent in adjacent ones. On the inner side of the nuclear envelope of many cell

types is present fibrous material which has been called the fibrous lamina which extends into the nucleoplasm.

Function of Nuclear Envelope:

The nuclear envelope is an interface between nucleus and the cytoplasm. It serves to separate the genetic component of the cell (the chromosomes) from the protein-synthesis machinery (ribosome and ER). It thus provides protection to DNA against the mutagenic effects of cytoplasmic enzymes. It is concerned with nucelocytoplasmic exchange, attachment of structural elements to the cytoplasm, attachment of nuclear components, contribution to other cell membranes and electron transport activity.

Assembly and Disassembly of Nuclear Membrane:

From the light microscopic observation, it is known that the nuclear envelope disappears in late prophase during cell division when the chromatin condenses into chromosome and reappears around the daughter chromosomes during telophase. Disappearance and reappearance of nuclear membrane during cell division are also correlated with disassembly and assembly of nuclear lamina.

Immunofluorescence studies have shown that at the onset of prophase the lamins start to dis-assembly and appear in the cytoplasm. Disassembly of the lamina takes place due to de-polymerisation. Biochemical studies have shown that the de-polymerisation of lamina is due to phosphorylation. The phosphorylation reaction is catalysed by an enzyme, lamin kinase. This whole process, in turn, causes the nuclear envelope to disassembly into a number of small vesicles that disperse into the cytoplasm. The electron microscope, however, shows that lamin B remains attach with these vesicles, whereas lamins A and C are de-polymerised to small oligomer and dispersed into the cytoplasm.

During telophase, after the daughter chromosomes have separated and begin to de-condense, nuclear assembly is started. This process induces the de-phosphorylation and polymerisation of lamins into a fibrous network and simultaneously small nuclear vesicles fuse with each other. Polymerised lamin B associated with fused vesicle in turn binds with polymerised lamins A and C and the whole process makes a bridge between the chromatin and fused small vesicle of nuclear envelope.

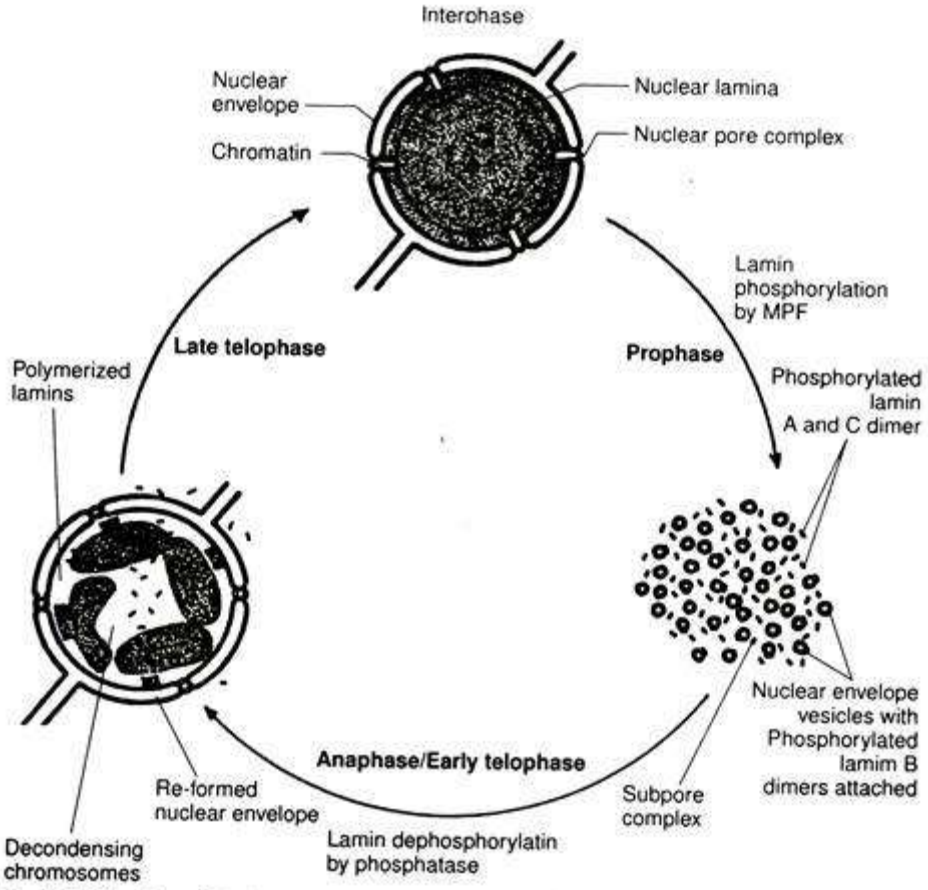


Fig. 9.7: Overview of depolymerisation and polymerisation of nuclear lamins during the cell cycle.

Structure of Nuclear Pore Complex :

The nuclear pore is a large complex structure of 125 million Daltons with 120 nm diameter and 50 nm thickness. Electron micrograph has shown that nuclear pore complexes have an eight-fold symmetry. Pore complex consists of annuli and a structure is formed from a set of large protein granules arranged in octagonal patterns.

The hole in the centre of each complex often appears to be plugged by a large central granule. Eight radial spokes also extends from plug to rings (Fig. 2.67 & 2.68).

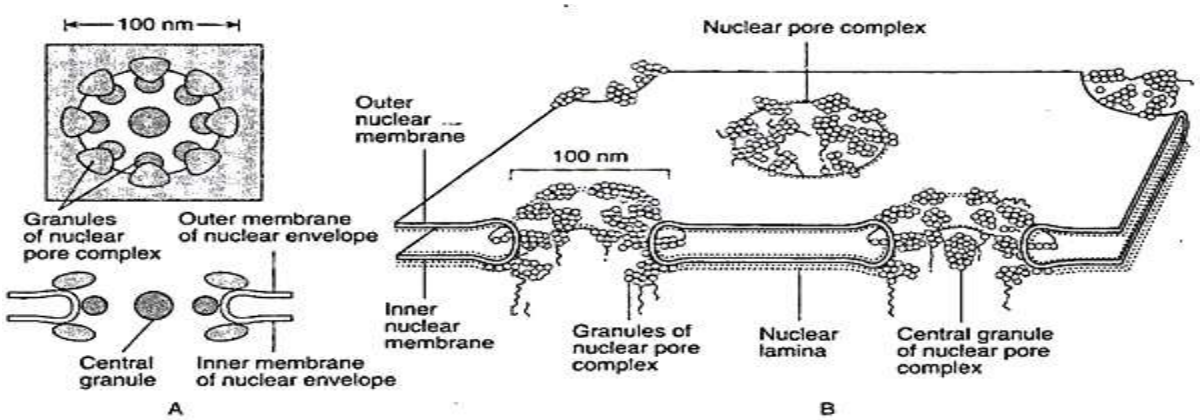


Fig. 2.67: The arrangement of the nuclear pore complexes in the nuclear envelope. A. A top view and a central vertical section. The "central granule" is seen in some pores but not in others; these granules may be a part of the pore, or they may be large complexes caught in transit through it. B. Three-dimensional sketch of a small region of the nuclear evelope (from Roy and De)

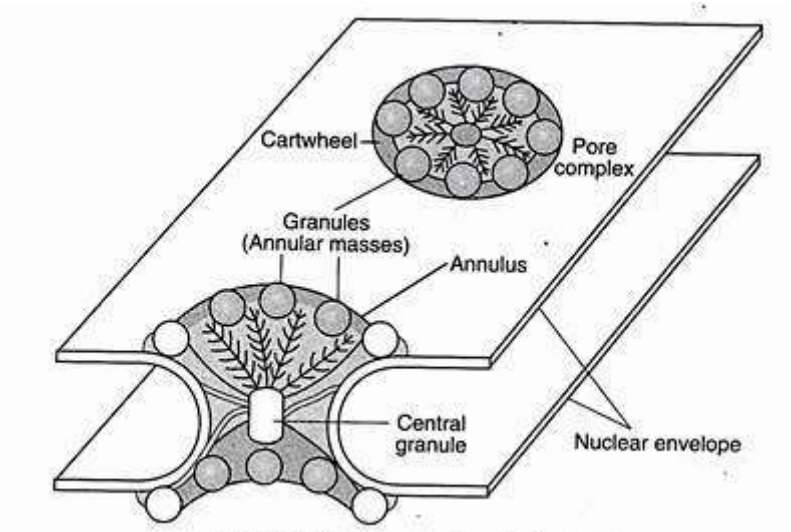


Fig. 2.68: The pore complex, Franke (1970)

During 1990s, significant progress has been made towards better understanding of the structure and function of tine nuclear pore. New pore protein have been identified, cloned, mutants isolated and detailed mechanism of nucleocytoplasmic transfer has been proposed.
Further, the pore has been reconstituted in vitro, a number of signal sequences and one or more signal sequence receptors have been identified and a new 'basket like structure' has been attached to the inner side of the nuclear pore.

It consists of four separate elements:
(i) Scaffold, which included majority of the pore,
(ii) Transporter, the central hub which carries out active transport (both import and export) of proteins and RNAs,
(iii) Short thick filaments attached to the cytoplasmic side of the pore,
(iv) A basket attached to the nucleocytoplasmic side of the pore (Fig. 2.69).
The scaffold is a stack of three closely apposed rings — cytoplasmic ring, nucleocytoplasmic ring and a central ring of thick spokes. The spokes of central ring are attached to the transporter on the inner side and to the nucleocytoplasmic and cytoplasmic rings on the outer side. Interspersed between the spokes are aqueous channels, 9 nm wide, which allow diffusion of proteins and metabolites between the nucleus and the cytoplasm.

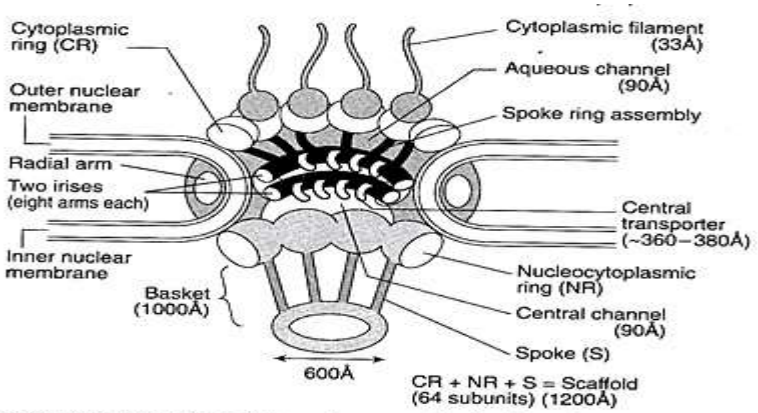


Fig. 2.69: Detailed structure of the nuclear pore, showing iris and transporter

The transporter is a proteinaceous ring, 36-38 nm in diameter and consists of two irises of eight arms each. The two irises are assumed to be stacked atop one another and open sequentially, each like the diaphragm of a camera, to let a nuclear protein or RNA pass through from the nucleus to the cytoplasm. On the cytoplasmic side of the pore, thick filaments of 3.3 nm in diameter, extend into the cytoplasm. On the nuclear side, a large basket like structure is found, which consists of eight filaments of 100 nm long, extending from nucleocytoplasmic ring of the pore and meeting a smaller ring of 60 nm in diameter within the nucleus. This basket plays an important role in RNA export.
The function of nuclear pore complex is the nucleocytoplasmic transport mediated through a number of proteins, called nucleoporin (NUP). The nuclear pore complex has a passive diffusion channel and also can diffuse many substances by active process using energy or signal sequence mediated by carrier molecules.

(a) Import of nuclear proteins through nuclear pore involves the formation of NLS- Protein-lmportin complex (NLS = nuclear localization sequence).

(**b)** Export of RNA from the nucleus across the pore is mediated through NES-Rev protein (NES = nuclear export sequence).

(c) Export followed by reimport of 5SrRNA and UsnRNA occurs through the nuclear pore by the protein with NES like sequence.

Transport across Nuclear envelope:
The entry and exit of large molecules from the cell nucleus is tightly controlled by the nuclear pore complexes (NPCs). Although small molecules can enter the nucleus without regulation macromolecules such as RNA and proteins require association with transport factors like karyopherins called importins to enter the nucleus and exportins to exit.
Protein that must be imported to the nucleus from the cytoplasm carry nuclear localization signals (NLS) that are bound by importins. A NLS is a sequence of amino acids that acts as a tag. They are diverse in their composition and most commonly hydrophilic, although hydrophobic sequences have also been documented. Proteins, transfer RNA, and assembled ribosomal subunits are exported from the nucleus due to association with exportins, which bind signaling sequences called nuclear export signals (NES). The ability of both importins and exportins to transport their cargo is regulated by the small Ras related GTPase, Ran.
GTPases are enzymes that bind to a molecule called guanosine triphosphate (GTP) which they then hydrolyze to create guanosine diphosphate (GDP) and release energy. Ran is in a different conformation depending on whether it is bound to GTP or GDP. In its GDP bound state, Ran is capable of binding karyopherins (importins and exportins). Importins release cargo upon binding to RanGTP, while exportins must bind RanGTP to form a ternary complex with their export cargo. The dominant nucleotide binding state of Ran depends on whether it is located in the nucleus (RanGTP) or the cytoplasm (RanGDP).

Nuclear import:

Importin proteins bind their cargo in the cytoplasm, after which they are able to interact with the nuclear pore complex and pass through its channel. Once inside the nucleus, interaction

with Ran-GTP causes a conformational change in the importin that causes it to dissociate from its cargo. The resulting complex of importin and Ran-GTP then translocates to the cytoplasm, where a protein called Ran Binding Protein (RanBP) separates Ran-GTP from importin. Separation allows access to a GTPase activating protein (GAP) that binds Ran-GTP and induces the hydrolysis of GTP to GDP. The Ran-GDP produced from this process now binds the nuclear transport factor NUTF2 which returns it to the nucleoplasm. Now in the nucleus, the Ran-GDP interacts with a guanine nucleotide exchange factor (GEF) which replaces the GDP with GTP, resulting again in Ran-GTP, and beginning the cycle anew.

Nuclear export:

Nuclear export roughly reverses the import process; in the nucleus, the exportin binds the cargo and Ran-GTP and diffuses through the pore to the cytoplasm, where the complex dissociates. Ran-GTP binds GAP and hydrolyzes GTP, and the resulting Ran-GDP complex is restored to the nucleus where it exchanges its bound ligand for GTP. Hence, whereas importins depend on RanGTP to dissociate from their cargo, exportins require RanGTP in order to bind to their cargo. A specialized mRNA exporter protein moves mature mRNA to the cytoplasm after post-transcriptional modification is complete. This translocation process is actively dependent on the Ran protein, although the specific mechanism is not yet well understood. Some particularly commonly transcribed genes are physically located near nuclear pores to facilitate the translocation process. TRNA export is also dependent on the various modifications it undergoes, thus preventing export of improperly functioning tRNA. This quality control mechanism is important due to tRNA's central role in translation, where it is involved in adding amino acids to a growing peptide chain. The tRNA exporter in vertebrates is called exportin-t. Exportin-t binds directly to its tRNA cargo in the nucleus, a process promoted by the presence of RanGTP. Mutations that affect tRNA's structure inhibit its ability to bind to exportin-t, and consequentially, to be exported, providing the cell with another quality control step. As described above, once the complex has crossed the envelope it dissociates and releases the tRNA cargo into the cytosol.

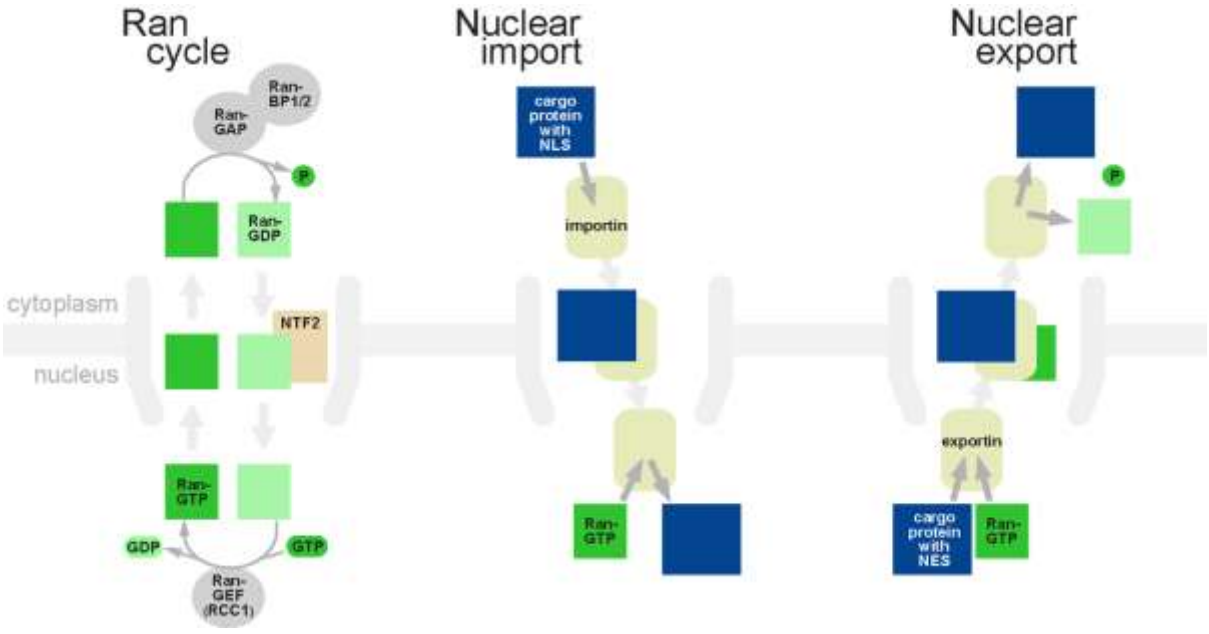


Figure : Macromolecules, such as RNA and proteins, are actively transported across the nuclear membrane in a process called the Ran-GTP nuclear transport cycle.

Protein Shuttling:

Many proteins are known to have both NESs and NLSs and thus shuttle constantly between the nucleus and the cytosol. In certain cases one of these steps (i.e., nuclear import or nuclear export) is regulated, often by post-translational modifications. Protein shuttling can be assessed using a heterokaryon fusion assay

Regulation of nuclear transport:

The nuclear envelope establishes an essential regulatory barrier, which eukaryotic cells can use to control cellular processes such as gene expression and cell cycle progression. Thus, the dynamic compartmentalization of proteins between the nucleus and the cytoplasm can be utilized to spatially and temporally regulate protein function. Use of this nucleocytoplasmic compartmentalization as a method for regulating cellular processes requires rapid, selective, and highly regulated nuclear transport.

All macromolecules that move into and out of the nucleus are transported through nuclear pore complexes, large proteinaceous channels that are embedded in the nuclear envelope. Soluble factors are required to recognize, target, and transport most macromolecules through the nuclear pores. The best characterized nuclear transport process occurs via receptor recognition of classic nuclear localization signals (NLSs)1 on protein cargoes targeted for nuclear import (3, 6). These classic NLS cargo proteins are recognized in the cytoplasm by a heterodimeric receptor composed of importin/karyopherin α and β. Importin α recognizes and binds the NLS, and importin β translocates the trimeric import complex through the nuclear pore (3, 5). Delivery into the nucleus is dependent on the small GTPase Ran, which governs the interactions between the nuclear transport receptors and macromolecular cargoes and thus confers directionality to nucleocytoplasmic transport. Once inside the nucleus, the cargo is delivered and the transport receptors are recycled to the cytoplasm.

Classic NLSs are typified by a single cluster of basic amino acids (monopartite) or two clusters of basic amino acids separated by a 10- to 12-amino acid linker (bipartite). The prototypical monopartite NLS is that of the SV40 large-T antigen (PKKKRKV) and the prototypical bipartite NLS is that of nucleoplasmin (KRPAATKKAGQAKKKK). Recent studies that have examined the structural and energetic contributions made by individual amino acid residues within the sequence have refined our definition of a functional NLS and have thereby allowed easier identification of potential NLS signals within a protein sequence

The dynamic compartmentalization of NLS-containing proteins requires regulated changes in the relative import and/or export rates of a protein. In numerous cases, transport of an NLS cargo into the nucleus is regulated by phosphorylation . There are at least three ways in which phosphorylation could regulate protein import into the nucleus: 1) phosphorylation could cause a conformational change in the protein, which reveals or masks an NLS sequence; 2) phosphorylation could cause the release or binding of an NLS masking protein; or 3) phosphorylation could directly modulate the affinity of an NLS for the import receptor importin α. Examples of each of these mechanisms include the growth regulatory protein STAT (signal transducers and activators of transcription) where phosphorylation causes the protein to dimerize creating an NLS, the p65 subunit of NF-κB where phosphorylation of NF-κB-bound

I-κB leads to degradation of I-κB and unmasking of the NF-κB NLS, and the v-Jun oncoprotein where phosphorylation may directly modulate interactions with the NLS receptor . Direct regulation of the interaction between an NLS cargo and importin α (regulation method 3 above) requires that the phosphorylation site is within or adjacent to the NLS. In contrast, the other two forms of regulation, methods 1 and 2 above, could be due to phosphorylation at any site within the protein. Each of these modes of regulation is non-exclusive, and thus a combination could regulate the nuclear transport of a particular cargo. Here we will focus on the direct modulation of importin α binding by phosphorylation.

A number of important regulatory proteins contain phosphorylation sites located within or adjacent to classic NLS sequences. Recent studies have shown that transport into and out of the nucleus correlates with regulated phosphorylation at these sites. For example, p53, the adenomatous polyposis protein (APC), and the *Saccharomyces cerevisiae* Swi6 protein all have documented phosphorylation sites within or proximal to their NLS sequences. Phosphorylation of these sites is associated with cytoplasmic protein localization and, conversely, hypophosphorylation correlates with nuclear protein localization. Thus, phosphorylation appears to represent an important mechanism to regulate the nuclear transport and consequently the function of an NLS cargo protein. Furthermore, the proximal position of the phosphorylation site to the NLS sequence suggests that the nuclear import of these cargoes may be modulated by directly regulating the binding affinity of the NLS for the NLS receptor.

Importin α structural studies revealed the determinants for specific recognition of classic NLS sequences by the NLS-binding pocket of importin α. Structures of the NLS binding domain of *S. cerevisiae* importin α (amino acid residues 89–530) bound to various NLS peptides showed that this domain of importin α consists of ten helical repeats known as armadillo motifs. These armadillo motifs form a concave NLS binding groove, which is lined by conserved tryptophan and asparagine residues and surrounded by acidic amino acids. This structure creates specific binding pockets for NLS cargoes that combine both hydrophobic interactions and electrostatic interactions with the positively charged residues of the NLS. These observations suggest that the addition of a negatively charged phosphate group proximal to an NLS could decrease binding of the NLS to importin α by disrupting the electrostatic interactions. Furthermore, a similarly positioned negative group within any classic NLS sequence may change its binding affinity for importin α and thus modulate the intracellular localization of a cargo protein.

Recent studies that correlate differences in the rate of import with changes in the phosphorylation state of NLS-containing proteins have not examined the change in binding affinity between the NLS and its receptor using a quantitative assay. Furthermore, although one of these studies solved the co-crystal structure of a phosphorylated peptide containing an NLS sequence and non-autoinhibited importin α, the phosphorylation site was 14 amino acids upstream of the NLS sequence. In contrast, in our analyses the phosphorylation sites examined are located one amino acid upstream of the NLS. Thus, it is not known to what extent phosphorylation proximal to or within an NLS changes the binding affinity for importin α. Furthermore, it is important to determine if this change in affinity is sufficient to account for the observed changes in protein localization. A complete understanding of phosphorylation-mediated regulation of nuclear import by modulation of the interaction between an NLS and importin α requires a quantitative model for the import of a cargo that correlates the in vitro interaction energies with the in vivo localization of a protein.

Translation (Protein Synthesis):

Proteins are giant molecules formed by polypeptide chains of hundreds to thousands of amino acids. These polypeptide chains are formed by about twenty kinds of amino acids. An amino acid consists of a basic amino group ($-NH_2$) and an acidic carboxyl group (-COOH). Different arrangement of amino acids in a polypeptide chain makes each protein unique.
Proteins are fundamental constituents of protoplasm and building material of the cell.

They take part in the structural and functional organization of the cell. Functional proteins like enzymes and hormones control the metabolism, biosynthesis, energy production, growth regulation, sensory and reproductive functions of the cell. Enzymes are catalysts in most of the biochemical reactions. Even the gene expression is controlled by enzymes. The replication of DNA and transcription of RNA is controlled by the proteinous enzymes.

Components of Protein Synthesis:
Protein synthesis is governed by the genetic information carried in the genes on DNA of the chromosomes.

I. Amino Acids:
Proteins are the polymers of amino acids. Therefore, amino acids form the raw material for protein synthesis. The proteins of living organisms need about 20 amino acids as building blocks or monomers. These are available in the cytoplasmic matrix as an amino acid pool.

II. DNA as Specificity Control:
A cell, in order to maintain its own special characteristics, must manufacture proteins exactly similar to those present already in it. Thus, protein synthesis requires specificity control to provide instructions about the exact sequence in which the given numbers and kinds of amino acids should be linked to get the desired polypeptides. The specificity control is exercised by DNA through mRNA sequences of 3 consecutive nitrogenous bases in the DNA double helix form the biochemical or genetic code. Each base triplet codes for a specific amino acid. Since the DNA is more or less stable, the proteins formed in a cell are exactly like the preexisting proteins.

III. RNAs:
RNA molecule is a long, un-branched, single-stranded polymer of ribonucleotides (Fig. 7.12). Each nucleotide unit is composed of three smaller molecules: a phosphate group, a 5- carbon ribose sugar, and a nitrogen-containing base. The bases in RNA are adenine, guanine, uracil and cytosine. The various components are linked up as in DNA. There are three types of RNA in every cell: messenger RNA or mRNA, ribosomal RNA or rRNA and transfer RNA or tRNA. The three types of RNAs are transcribed from different regions of DNA template, RNA chain is complementary to the DNA strand which produces it. All the three kinds of RNAs play a role in protein synthesis.

(a) mRNA:
The DNA, that controls protein synthesis, is located in the chromosomes within the nucleus, whereas the ribosomes, on which the protein synthesis actually occurs, are placed in the

cytoplasm. Therefore, some sort of agency must exist to carry instructions from the DNA to the ribosomes. This agency does exist in the form of mRNA.

The mRNA carries the message (information) from DNA about the sequence of particular amino acids to be joined to form a polypeptide, hence its name. It is also called informational RNA or template RNA. The mRNA forms about 5% of the total RNA of a cell. Its molecule is linear and the longest of all the three RNA types. Its length is related to the size of the polypeptide to be synthesized with its information.

There is a specific mRNA for each polypeptide. Because of the variation of size in mRNA population in a cell, the mRNA is often called heterogeneous nuclear RNA, or hnRNA:

In eukaryotes, mRNA carries information for one polypeptide only It is monocistronic (monogenic) because it is transcribed from a single cistron (gene) and has a single initiator codon and a single terminator codon.

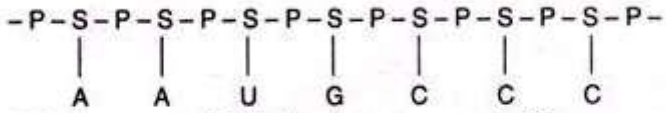


Fig. 7.12 A segment of RNA showing arrangement of its components.

Bacterial mRNA often carries information for more than one polypeptide chains. Such a mRNA is said to be polycistronic (polygenic) because it is transcribed from many contiguous (adjacent) genes. A polycistronic mRNA has an initiator codon and a terminator codon for each polypeptide to be formed by it.

(b) tRNA:

The tRNA has many varieties. Each variety carries a specific amino acid from the amino acid pool to the mRNA on the ribosomes to form a polypeptide, hence its name. The tRNAs form about 15% of the total RNA of a cell. Its' molecule is the smallest of all the RNA types.

Structure of tRNA:

The universally accepted 2-dimensional model of tRNA is the "clover leaf model" formed due to pairing of short complementary sequences and the formation of unpaired loops (Fig. 4.2). The 5′ P end terminates usually into guanine (G), while the 3′ OH end always terminates into a 5′ CCA3′ sequence. Amino acid is carried on the 3′-end, associated with the adenine (A), general structure of clover leaf model is described below.

(1) The 3′-end terminates into 5'CCA3′ sequence that is always unpaired. The terminal A residue is the site at which the amino acid is bound covalently.

(2) Starting from the 3′-end, after the 3′ ACC5′ sequence, the region includes few paired bases.

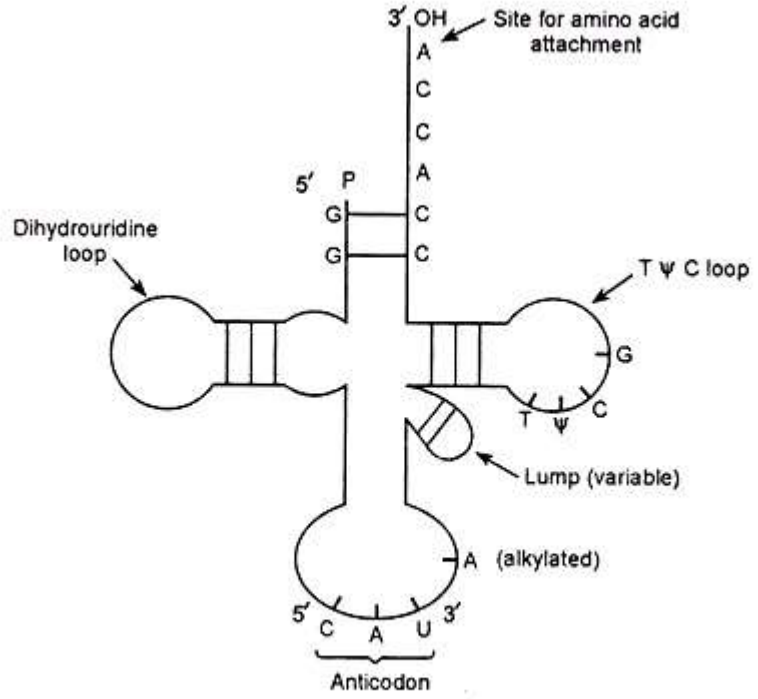


Fig. 4.2. Diagrammatic representation of the clover leaf model of transfer RNA. (This is a model of $tRNA^{met}$).

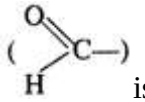
(3) Then comes the first loop containing 7 unpaired bases. This loop is designated as **"**TΨ C loop" because it always contains a sequence 5′ ribothymidine- pseudouridine- cytidine 3′. This loop is involved in binding to ribosome.

(4) After the "5′ -T Ψ C-3′ loop", in the 5′ direction, there occurs a loop of variable size, called the extra loop or the "lump". The lump may contain 3 to 21 bases.

(5) The third loop contains 7 unpaired bases and it has the "anticodon." Anticodon consists of 3 bases. At the 3′ -end of the anticodon, there is a purine (A or G) while at the 5′ -end, there is always a uracil (U). At the time of protein synthesis, anticodon pairs with its complementary "codon" on mRNA.

(6) The fourth loop is larger than others and contains 8-12 unpaired bases. It is designated as "D-loop" because it is rich in dihydrouridine (UH_2). The enzyme aminoacylsynthetase binds to this loop.

Initiator Transfer RNA:

Marcker and Sanger in 1964 isolated two different types of methionine specific tranfer RNAs. One type, designated as $tRNA_f^{met}$ carries the amino acid N-formyl methionine, while the other type, designated as $tRNA^{met}$ carries the normal methionine. After the formation of aminoacyl-tRNA (methionyl-tRNA), the formate group  is added to methionine to form N-formyl methionine, by the enzyme transformylase. The formate group comes from N-formyltetrahydrofolic acid.

The formate group protects the polypeptide chain from peptide bond formation with other amino acids. In prokaryotes the initiator amino acid is the N-f-methionine, while in eukaryotes,

it is the normal methionine. The initiator tRNA in eukaryotes also differs from the $tRNA^{met}$ and is designated as $_tRNA_f^{met}$.

tRNAs and their modifications:

Transfer RNAs (tRNAs), also called **"soluble RNAs"** (sRNAs) are small molecules varying from 75 to 100 nucleotides in length. They carry amino acids and bring them to ribosomes to form the polypeptide chain. There are 20 amino acids but the number of tRNA types is greater because some amino acids are adopted by more than one form of tRNAs.

Transfer RNAs are designated by the amino acid abbreviation in super script, such as, $tRNA^{gly}$ (tRNA for glycine). Different tRNA adopting the same amino acid are designated by number in subscript, e.g. $tRNA_1^{leu}$, $tRNA_2^{leu}$. Such tRNAs are called **"**iso-accepting tRNAs". Transfer RNAs are produced on DNA templates and the reaction is catalysed by DNA dependent RNA polymerase. Precursor tRNA is produced first and then processing is made to produce the mature tRNA. During processing, modifications of some bases occurs, such as dihydrouridine (UH_2), pseudouridine (Ψ), inosine (I), 7-methylguanosine (m^7 G), acetyl cytidine (acC), N^2-dimethyl guanosine (m_2G) and 1- methyl adenosine (m^1A) etc. About 10-15% of the bases in tRNA are modified.

(c) rRNA:

The rRNA molecule is greatly coiled. In combination with proteins, it forms the small and large subunits of the ribosomes, hence its name. It forms about 80% of the total RNA of a cell. The rRNA also seems to play some general role in protein synthesis.

(IV) Ribosomes:

Ribosomes serve as the site for protein synthesis. The small and large subunits of ribosomes occur separately when not involved in protein synthesis. The two sub units form association (join) when protein synthesis starts, and undergo dissociation (separate) when protein synthesis stops. Many ribosomes line up on the mRNA chain during protein synthesis. Such a group of active ribosomes is called a polyribosome, or simply a polysome.

In a polysome, the adjacent ribosomes are about 340 Å apart. The number of ribosomes in a polysome is related to the length of the mRNA molecule, which reflects the length of the polypeptide to be synthesized. It has been established that polypeptides are synthesized at the polysomes and not at the single free ribosomes as held earlier. This is true for both prokaryotes and eukaryotes as well as for the cell organelles such as mitochondria and plastids.

A ribosome has two binding sites for tRNA molecules. One is called A (acceptor or aminoacyl) site and the other is termed P (peptidyl) site. These sites span across the large and small subunits of the ribosome (Fig. 7.13). The A site receives the tRNA-amino acid complex. From P site, the tRNA leaves after leaving its amino acid to the forming polypeptide. However, the first tRNA-amino acid complex directly enters the P site of the ribosome.

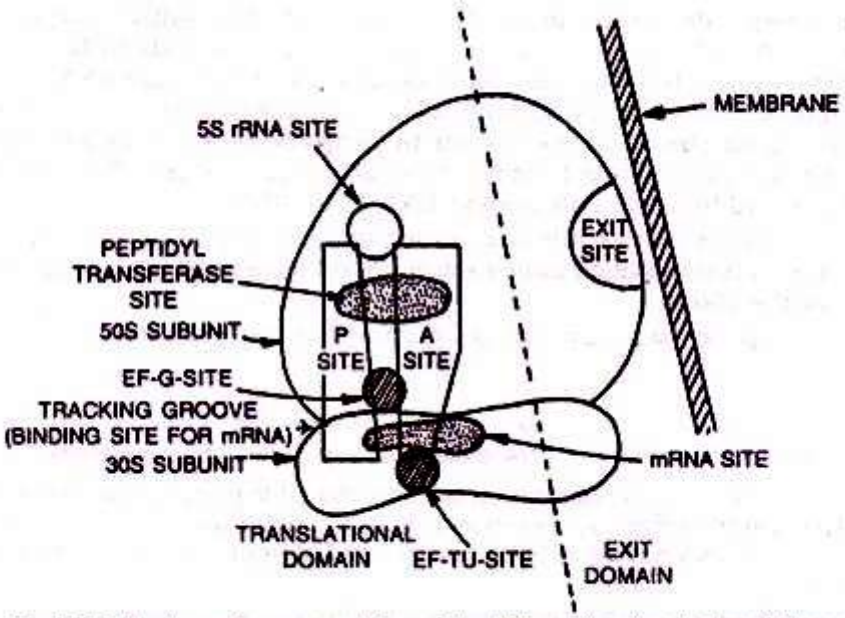


Fig. 7.13. A schematic representation of the different functional sites of ribosomes.

The function of the ribosome is to hold in position the mRNA, tRNA and the associated enzymes controlling the process until a peptide bond forms between the adjacent amino acids.

Mechanisms of Protein Synthesis:

In prokaryotes, the RNA synthesis (transcription) and protein synthesis (translation) take place in the same compartment as there is no separate nucleus. But in eukarytoes, the RNA synthesis takes place in the nucleus while the protein synthesis takes place in the cytoplasm. The mRNA synthesized in the nucleus is exported to cytoplasm through nucleopores.

First, Francis Crick in 1955 suggested and later Zemecnik proved that prior to their incorporation into polypeptides, the amino acids attach to a special adaptor molecule called tRNA. This tRNA has a three nucleotide long anticodon which recognizes three nucleotide long codon on mRNA.

Role of Ribosomes in Protein synthesis:

Ribosome is a macromolecular structure that directs the synthesis of proteins. A ribosome is a multicomponent, compact, ribonucleoprotein particle which contains rRNA, many proteins and enzymes needed for protein synthesis. Ribosome brings together a single mRNA molecule and tRNAs charged with amino acids in a proper orientation so that the base sequence of mRNA molecule is translated into amino acid 1 sequence of polypeptides.

Ribosome is a nucleoprotein particle having two subunits. These two subunits lie separately but come together for the synthesis of polypeptide chain. In E. coli ribosome is a 70S particle having two subunits of 30S and 50S. Their association and dissociation depends a upon the concentration of magnesium.

$$\underset{\text{Subunits of ribosome}}{30S} + 50S \underset{\text{Low magneisum conc.}}{\overset{\text{High magnesium conc.}}{\rightleftharpoons}} 70S \text{ ribosome}$$

Small subunit of ribosome contains the decoding centre in which charged tRNAs decode o the codons of mRNA. Large subunit contains peptidyl transferase centre, which forms the peptide bonds between successive amino acids of the newly synthesized peptide chain.

Both 30S and 50S subunits consist of ribosomal RNA (rRNA) and proteins. The mRNA binds to the 16S rRNA of smaller subunit. Near its 5′-end mRNA binds to the 3′-end of 16S rRNA. The main role of ribosome is the formation of peptide bond between successive amino acids of the newly synthesized polypeptide chain. The ribosome has two channels in it. The linear mRNA enters and escapes through one channel, which has the decoding centre. This channel is accessible to the charged tRNAs. The newly synthesized polypeptide chain escapes through the other channel.

Direction of Translation:

Each protein molecule has an -NH_2 end and -COOH end. Synthesis begins at amino end and ends at carboxyl end. The mRNA is translated in 5 → 3′ direction from amino to carboxyl end. Synthesis of mRNA from DNA transcription also occurs in 5′ → 3′ direction.

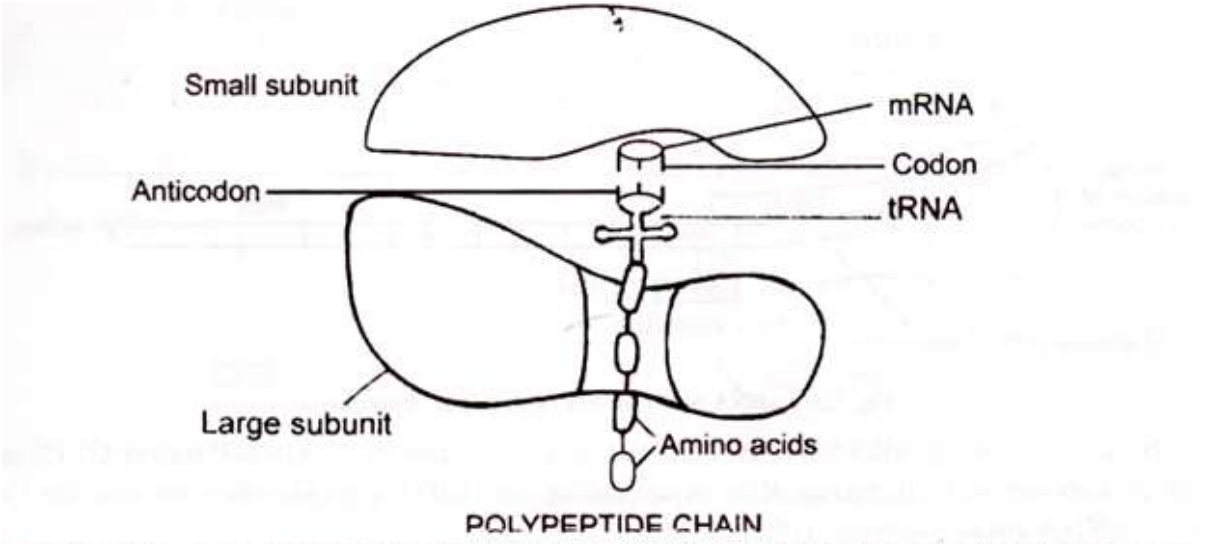


Fig. 12.1. Ribosome showing two subunits and position of mRNA and tRNA. The nascent polypeptide chain passes through a channel.

Steps of Protein Synthesis:

Protein biosynthesis involves following major steps:

(i) Activation of Amino Acids:

Amino acid reacts with ATP to form amino acid -AMP complex and pyrophosphate. The reaction is catalyzed by a specific amino acid-activating enzyme called aminoacyl- tRNA synthetase in the presence of Mg^{2+}. There is a separate aminoacyl – tRNA synthetize enzyme for each kind of amino acid. Much of the energy released by the separation of phosphate groups from ATP is trapped in the amino acid — AMP complex.

The complex remains temporarily associated with the enzyme. The amino acid-AMP-enzyme complex is called an activated amino acid (Fig. 7.14). The pyrophosphate is hydrolysed to 2Pi, driving the reaction to the right.

$$\text{Amino acid} + \text{ATP} \xrightarrow{\text{Activating enzyme, } Mg^{2+}} \text{Amino acid- AMP-Enzyme complex} + PP_i$$

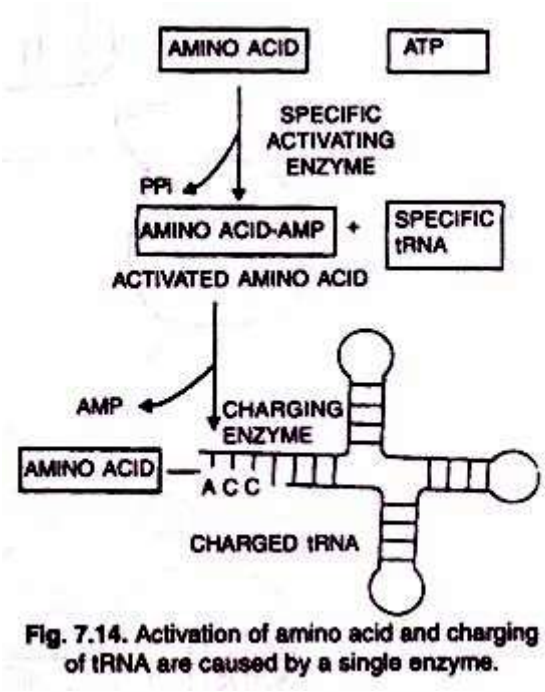


Fig. 7.14. Activation of amino acid and charging of tRNA are caused by a single enzyme.

(ii) Charging of tRNA:

The amino acid-AMP- enzyme complex joins to the amino acid binding site of its specific tRNA, where its -COOH group bonds to – OH group of the terminal base triplet CCA. The reaction is catalyzed by the same aminoacyl-tRNA synthetase enzyme. The resulting tRNA-amino acid complex is called a charged tRNA (Fig. 7.14). AMP and enzyme are freed. The freed enzyme can activate and attach another amino acid molecule to another tRNA molecule. The energy released by change of ATP to AMP is retained in the amino acid-tRNA complex. This energy is later used to drive the formation of peptide bond when amino acids link together on ribosomes.

$$\text{Amino acid-AMP-Enzyme Complex + tRNA} \xrightarrow[\text{Synthetase}]{\text{Aminoacyl - tRNA}} \text{tRNA - Amino Acid Complex + AMP + Enzyme}$$

The tRNA-amino acid complex moves to the site of protein synthesis, the ribosome.

(iii) Activation of Ribosomes:

The small and the large subunits of ribosomes must be joined together for protein synthesis. This is brought about by mRNA chain. The latter joins the small ribosomal subunit by first codon through base pairing with appropriate sequence on rRNA. The combination of the two is called initiation complex (Fig. 7.15). The large subunit later joins the small subunit, forming active ribosome. Activation of ribosome by mRNA requires proper concentration of Mg^{2+} (0.001 Molar conc.)

(iv) Assembly of Amino Acids (Polypeptide Formation):

The events in protein synthesis are better known in bacteria than in eukaryotes. Although these are thought to be similar in the two groups, some differences do occur. The following description refers mainly to protein synthesis in bacteria on the 70S ribosomes. Polypeptide formation involves 3 events: initiation, elongation and termination of amino acid chain.

(a) Initiation of Polypeptide Chain:

The mRNA chain has at its 5′ end an "initiator" or "start" codon (AUG) that signals the start of polypeptide formation. This codon lies close to the P site of the ribosome. The amino acid formyl-methionine (methionine in eukaryotes) initiates the process. It is carried by tRNA having UAC anticodon which bonds to AUG initiator codon of mRNA by hydrogen bonds.

Initiation factors (IF 1, IF 2 and IF 3) and GTP promote the initiation process. The large ribosomal subunit now joins the small subunit to complete the ribosome. At this stage, GTP is hydrolyzed to GDP. The ribosome has formylmethionine-bearing tRNA (tRNA f^{Met}) at the P site (Fig. 7.15). Later, the formylmethionine is changed to normal methionine by the enzyme deformylase. If not required, methionine is later separated from the polypeptide chain by a proteolytic enzyme amino peptidase. Initiation factors are used again to start new chains. As already established, translation of the codons of mRNA takes place in the 5′ – 3′ direction, thus P site and A site on the ribosomes recognize the polarity of the mRNA chain.

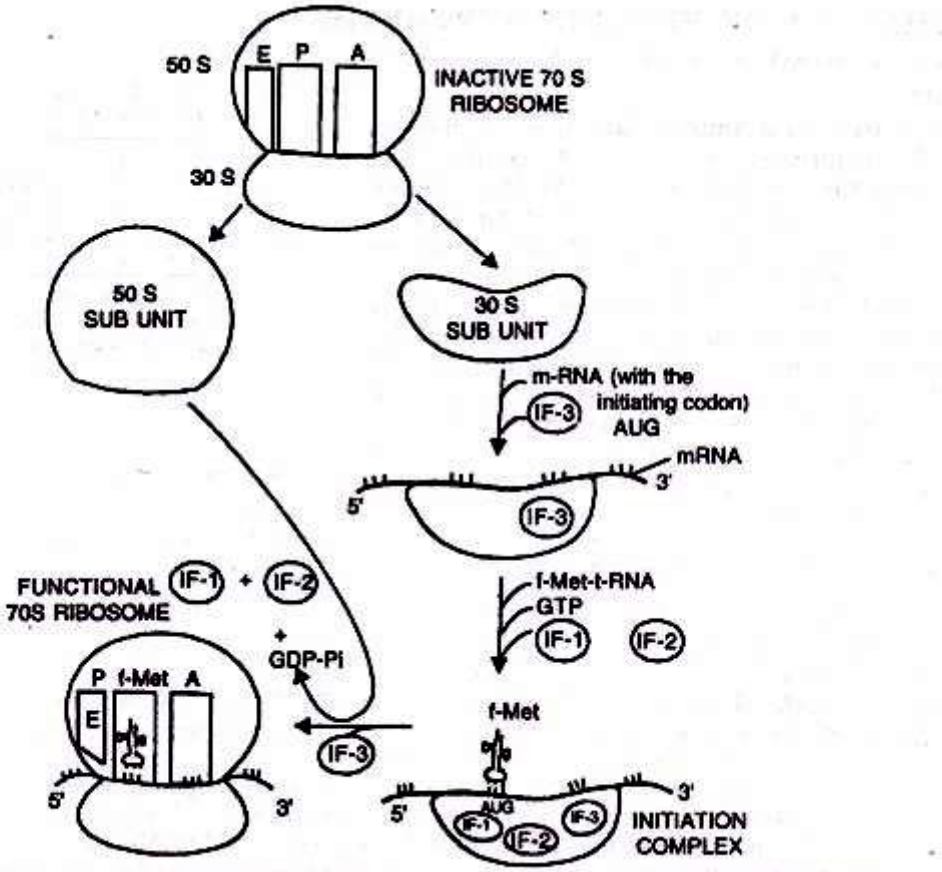


Fig. 7.15. Formation of initiation complex and assembly of the functional 70 S ribosome.

(b) Elongation Phase:

At this point fmet- $tRNA_f^{met}$ molecule in the 70S initiation complex occupies the P site on the ribosome. The other site for a tRNA molecule, i.e. the A site, is empty. The fmet-$tRNA_f^{met}$ is positioned in such a way that its anticodon pairs with the initiating AUG (or GUG) codon on mRNA. The reading frame is specified by this interaction and by pairing of the adjoining purine-rich sequence to a pyrimidine-rich sequence in 16S rRNA.

The elongation cycle in the protein synthesis begins with the insertion of an aminoacyl tRNA into the empty A site on the ribosome. The species of tRNA to be inserted depends upon the mRNA codon that is present in the A site. The complementary aminoacyl tRNA is transferred to the A site by a non-ribosomal specific cytoplasmic protein, called the elongation factor T (EF-T) that binds to the aminoacyl tRNA.

The factor EF-T contains two subunits, EF-Ts and EF-Tu. EF-Tu like IF2 contains a bound guanyl nucleotide and cycles between a GTP and a GDP. If the codon matches the anticodon, GTP is hydrolysed, positioning the aminoacyl tRNA in the A site and GDP bound with EF-Tu dissociates from the ribosome. A second elongation factor EF-Ts joins the EF-Tu complex and GDP is displaced from the complex forming a EF-Tu-Ts complex.
Finally, GTP binds to the EF-Tu- EF-Ts complex, releasing EF-Ts. EF-Tu containing bound GTP is ready to pick up another aminoacyl tRNA and deliver to the A site of the ribosome. This GTP-GDP cycle keeps repeating. It should be noted that EF-Tu does not recognise the fmet-tRNA initiator, hence the initiator tRNA is not delivered to the A site.
On the contrary before fmet-tRNAet, like all other aminoacyl tRNAs, can bind to EF-Tu. This explains why internal AUG codons are not read by initiator tRNA. It has been observed that rapid binding of EF-Tu to an activated aminoacyl-tRNA prevents hydrolysis, but after the formation of H'-Tu-GTP-tRNA complex, a time lag of several milliseconds allows the codon-anticodon mismatches to diffuse away (before OTP hydrolysis).

Peptide Bond Formation and Translocation:
Once the initiator fmet-tRNA occupies the P site and the next aminoacyl-tRNA occupies the A site, a peptide bond between the adjacent amino acids is formed by an enzyme, peptidyl transferase belonging to the 50S subunit. The active site of the peptidyl transferase is the 23 S rRNA. The uncharged $tRNA_f^{met}$ occupies the P site and the dipeptide formed is attached to the second tRNA occupying the A site following the formation of a peptide bond. The product of the first peptide bond formation is called dipeptidyl-tRNA bound to the A site.
The next step of the elongation cycle is translocation, which requires a third elongation factor EF-G (also called translocase) causing hydrolysis of GTP.

Three important movements occur:
(1) The fmet-tRNA which is now uncharged leaves the P site,
(2) The second tRNA with bound dipeptide is moved to the P site, and
(3) mRNA moves a distance of three nucleotides.
After translocation, the A site is opened up to accept the incoming aminoacyl-tRNA to match the next codon, now positioned at the A site for the next round of elongation (Fig. 7.16). The factor EF-Tu delivers the next aminoacyl-tRNA for the empty A site.
The accuracy of protein synthesis depends on having the correct aminoacyl-tRNA in the A site when the peptide bond is formed, hence the incoming aminoacyl-tRNA is meticulously scrutinized so that its anticodon is complementary and matches the codon at the A site. A mismatch aminoacyl- tRNA may bind with two or three nucleotides of a codon only temporarily, but will leave the A site before a peptide bond is formed. It takes a few milliseconds for the ribosome to decide if the incoming aminoacyl-tRNA is the correct one or not and the time lag is determined by GTPase site of EF-Tu. A peptide bond cannot be formed

until EF-Tu is released from the aminoacyl-tRNA and the process requires hydrolysis of GTP to GDP and Pi.

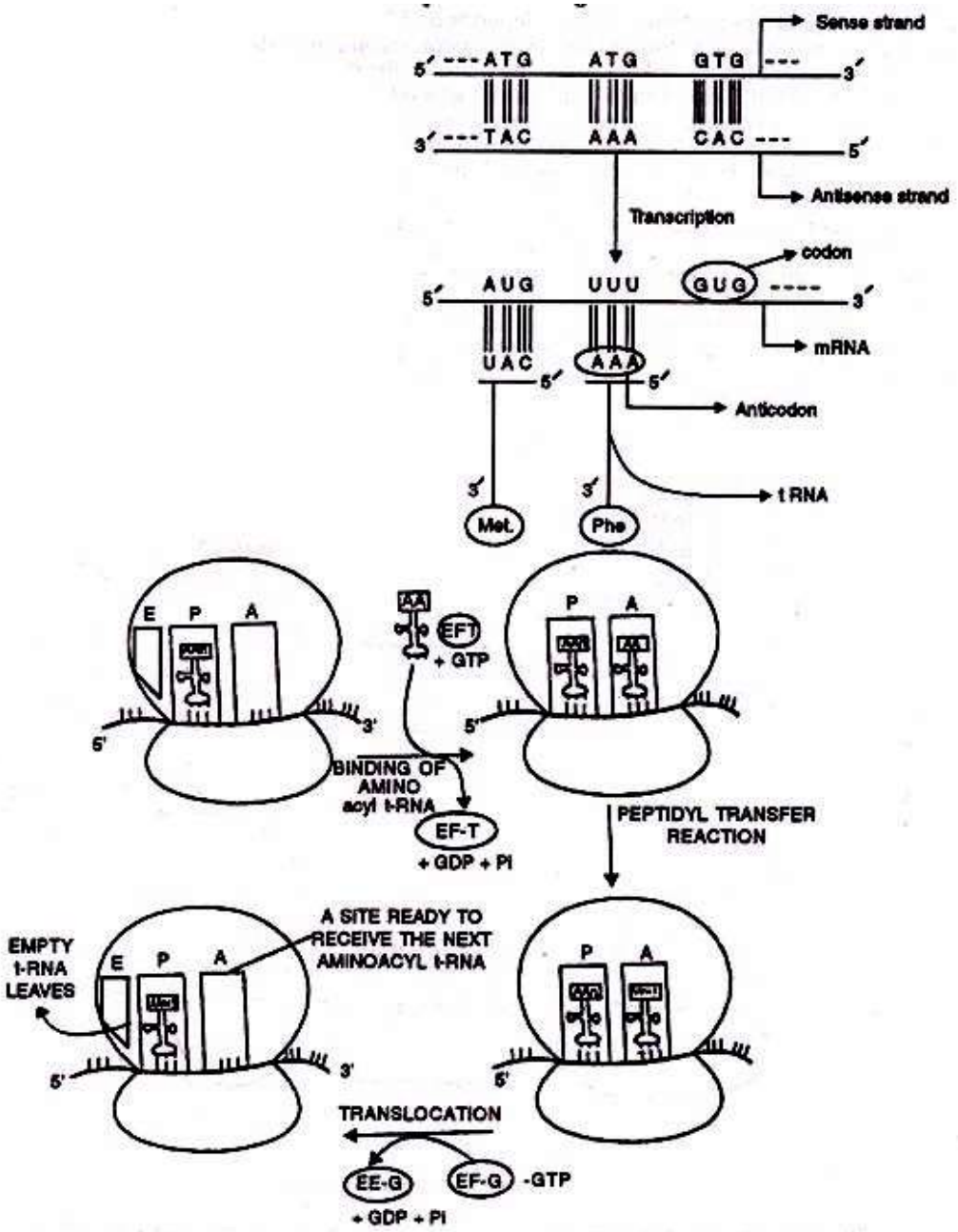


Fig. 7.16. The elongation process of peptide chain. AA n+1 denotes a formyl methionyl peptide.

Termination Phase:

Two conditions are necessary for termination of protein synthesis. One is the presence of a stop codon that signals the chain elongation to terminate, and the other is the presence of release factors (RF) which recognise the chain terminating signal. There are three terminating codons, UAA, UGA and UAG for which tRNAs do not exist. Termination of polypeptide chain is signalled by one of these codons in the mRNA. Behind all this complexity is the fact that after the polypeptide chain has reached its full length, its carboxyl end is still bound to its tRNA adapter.

Termination must, therefore, involve the splitting of the terminal tRNA. Release of the peptidyl tRNA from the ribosome is promoted by three specific release factors, RF1, RF2 and RF3. RF1

recognises triplets UAA and UAG, while RF2 recognises UAA and UGA. The third factor RF3 does not possess any release activity of its own, but it binds to OTP and stimulates the binding of RF1 and RF2 with the ribosome.

In E. coli, 16S rRNA is essential in reading the stop codon. The release factors bind to stop codon to cause a shift of the polypeptidyl-tRNA from A to P site (Fig. 7.17). Whether OTP hydrolysis is required for chain termination is not yet firmly established, although the RF3, which appears to enhance RF1 and RF2 binding with ribosome, does not bind to OTP.

The ester bond between the polypeptide chain and the last tRNA is then hydrolysed. Binding of RF to the terminating codon causes water to act as the acceptor of the growing peptide and not another amino acid on a tRNA Release of the polypeptide chain is followed by dissociation of mRNA and tRNA. Subsequently dissociation of 30S and SOS ribosome subunits takes place with concomitant binding of IF3 to 30S subunit to prevent reassembly in the absence of mRNA and fmet-tRNA.

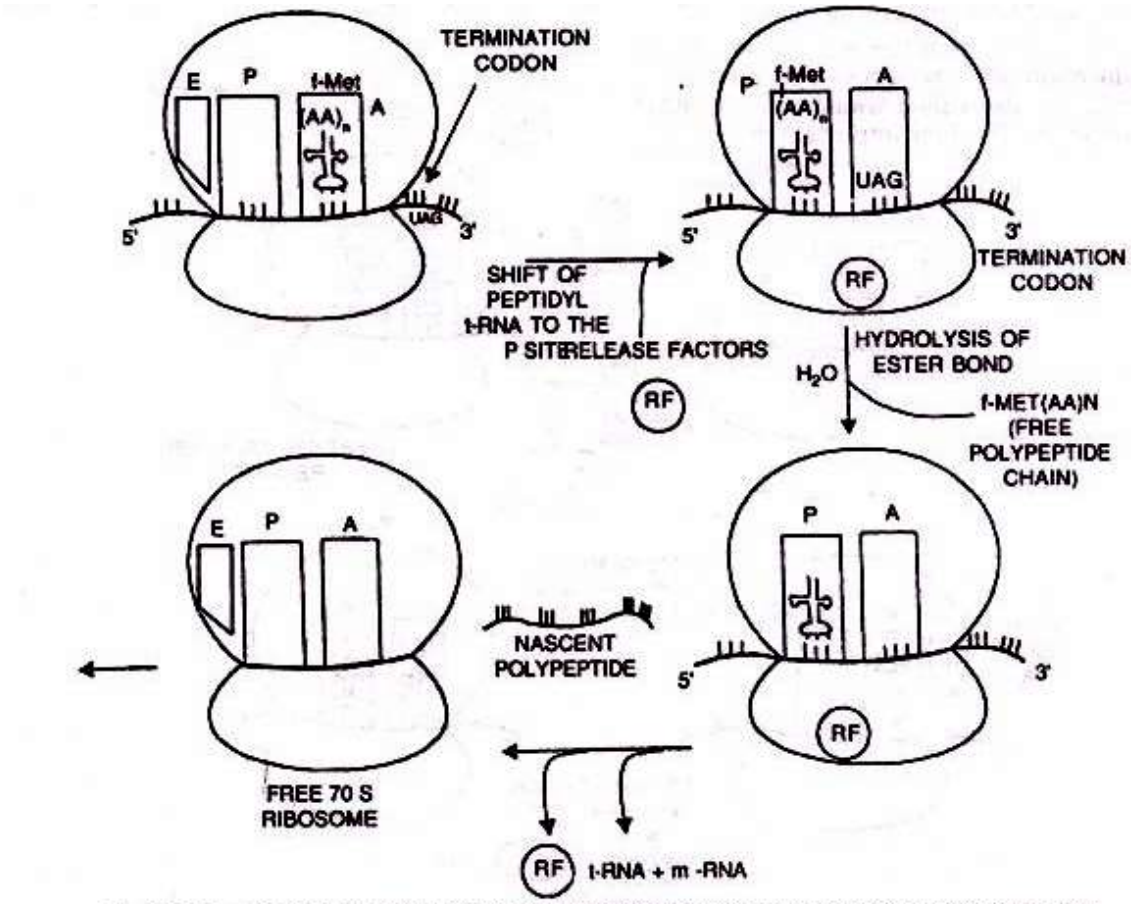


Fig. 7.17. Termination of protein synthesis. Steps showing the release of completed chain. The termination codon (UAG) at the A site alongwith the release factors helps in the termination process.

Polyribosome or Polysome:

A single mRNA molecule can be read simultaneously by several ribosomes. A polyribosome or polysome consists of several ribosomes attached to the same RNA. The number of ribosomes in a polysome depends upon the length of mRNA.

A fully active mRNA has one ribosome after every 80 nucleotides. There may be about 50 ribosomes in a polycistronic mRNA of prokaryotes. Ribosomes move along mRNA in 5′ 3′ direction. There is a gradual increase in the size of polypeptide chain as the ribosomes move

along mRNA towards its 3′-end. Polypeptide chain starts near the 5′-end and is completed near the 3′-end.

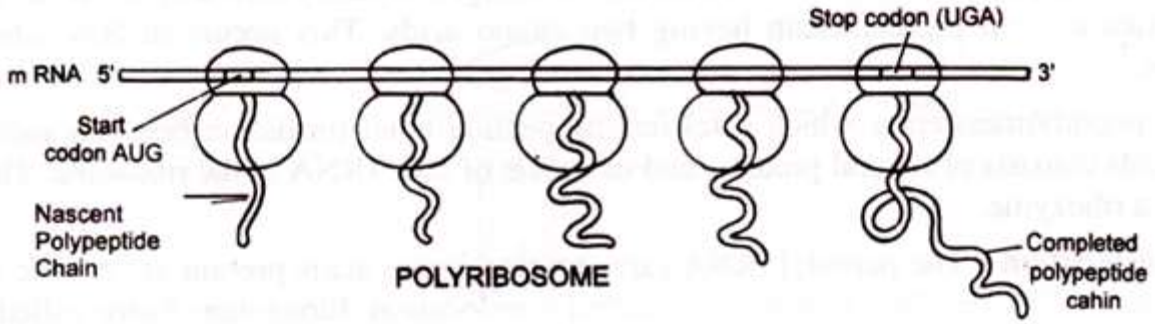


Fig. 12.9. Polyribosome

The ribosomes closest to the 5′-end of mRNA have the smallest polypeptide chain, while ribosomes nearest to the 3′-end have longest chain. Polysome increases the rate of protein synthesis tremendously. In bacteria protein is synthesized at the rate of about 20 amino acids per second.

Simultaneous Transcription and Translation in Prokaryotes:

In prokaryotes, all components of transcription and translation are present in the same compartment. The mRNA molecule is synthesized in 5′ → 3′ direction and protein synthesis also occurs in 5′ → 3′ direction. In this way mRNA molecule while still under synthesis has a free 5′-end whose other end is still under synthesis.

Ribosomes bind at free 5′-end and start protein synthesis. In this way the free end (5′-end) of mRNA starts the process of protein synthesis while still attached to DNA. This is called Coupled Transcription and Translation. This increases the speed of protein synthesis. After the protein synthesis is completed, the degradation of mRNA molecule by nucleases also starts at 5′-end and proceeds in 5′ → 3′ direction.

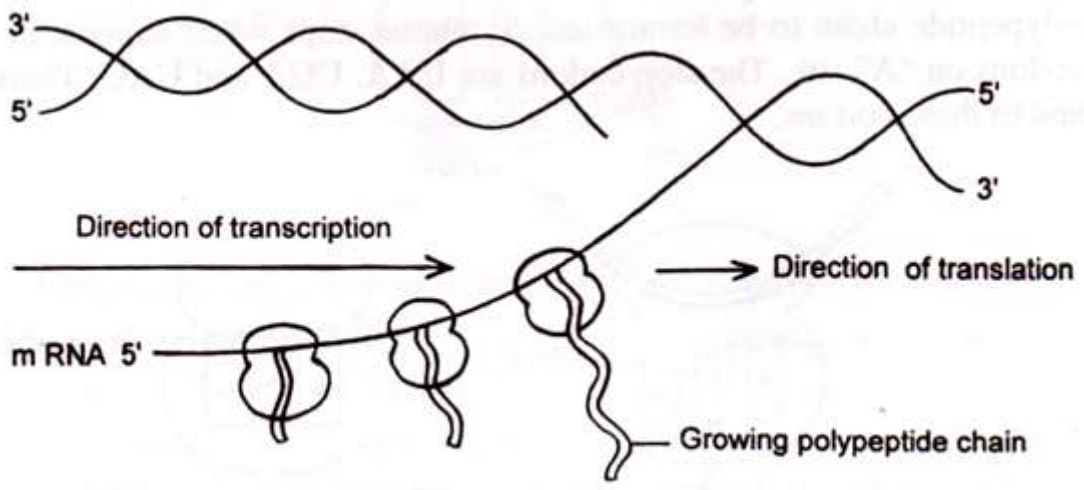


Fig. 12.10. Coupled transcription and translation.

Protein Synthesis in Eukaryotes:

Protein synthesis in eukaryotes is basically similar to that of prokaryotes except some differences. The ribosomes in eukaryotes are of 80S having 40S and 60S subunits. In eukaryotes the initiating amino acid is methionine and not f-methionine as in the case of prokaryotes. A special tRNA binds methionine to start codon AUG. This tRNA is called $tRNAi^{Met}$. This is distinct from $tRNA^{Met}$ which binds amino acid methionine to any other internal position in the polypeptide.

There is no Shine-Dalgarno sequence in eukaryotic mRNA to function as ribosome binding site. Between 5′-end and AUG codon of mRNA there is a sequence of bases called cap. Small subunit of ribosome scans the mRNA in 5′ → 3′ direction until it comes across 5′- AUG-3′ codon. This process is called scanning. Initiation factors also closely associated with 3′-end of mRNA through its poly-A tail. Initiation factors circularize mRNA by its poly-A tail. In this way poly-A tail also contributes to the translation of mRNA. Eukaryotic mRNAs are monocistsonic and encode a single polypeptide, therefore have a single open reading frame.

There are ten initiation factors in eukaryotes. They are elF (eukaryotic intiation factors) are elFI, eIF2, eIF3, eIF4A, eIF4B, eIF4C, eIF4D, eIF4F, eIF5, eIF6. There are two elongation factors in eukaryotes like prokaryotes. They are eEFl (similar to EF-Tu) and eEF2 (similar to EF-G). Eukaryotes have only one release factor eRF which requires GTP termination of protein synthesis. It recognizes all the three stop codons.

In eukaryotes the mRNA is synthesized in the nucleus, then processed, modified and passed on into the cytoplasm through nucleopores. The protein synthesis takes place in the cytoplasm. The mRNA in prokaryotes is very unstable and its life span is of a few minutes only. The mRNA of eukaryotes is quite stable and has a longer life span extending upto several days.

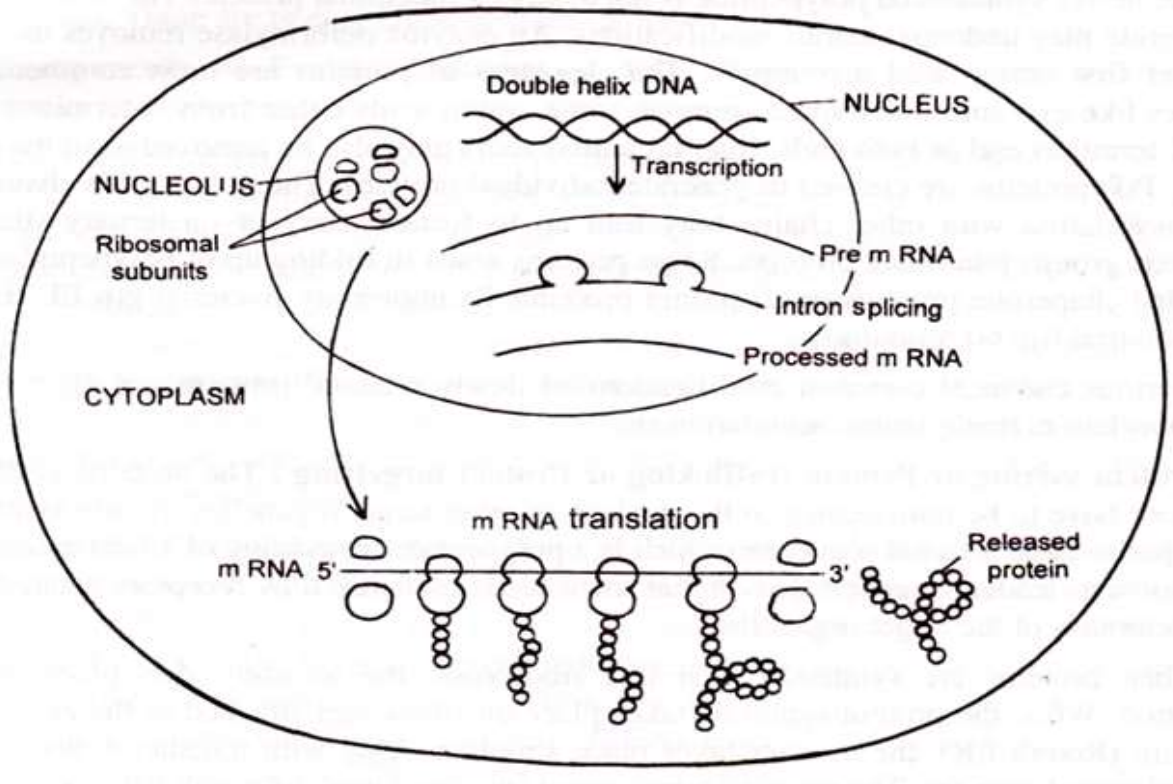


Fig. 12.11. Transcription, mRNA processing and mRNA translation.

Protein Synthesis on Bound Ribosomes:

Ribosomes occur in free state in the cytoplasm as well as bound to the outer surface of endoplasmic reticulum called rough endoplasmic reticulum (RER). The attachment of ribosomes to ER occurs after the protein synthesis starts. Whether the ribosomes synthesize protein on free or attached state depends upon the type of proteins to be synthesized by ribosomes. Most of the proteins which remain in free state in the cytoplasm are synthesized by free ribosomes.

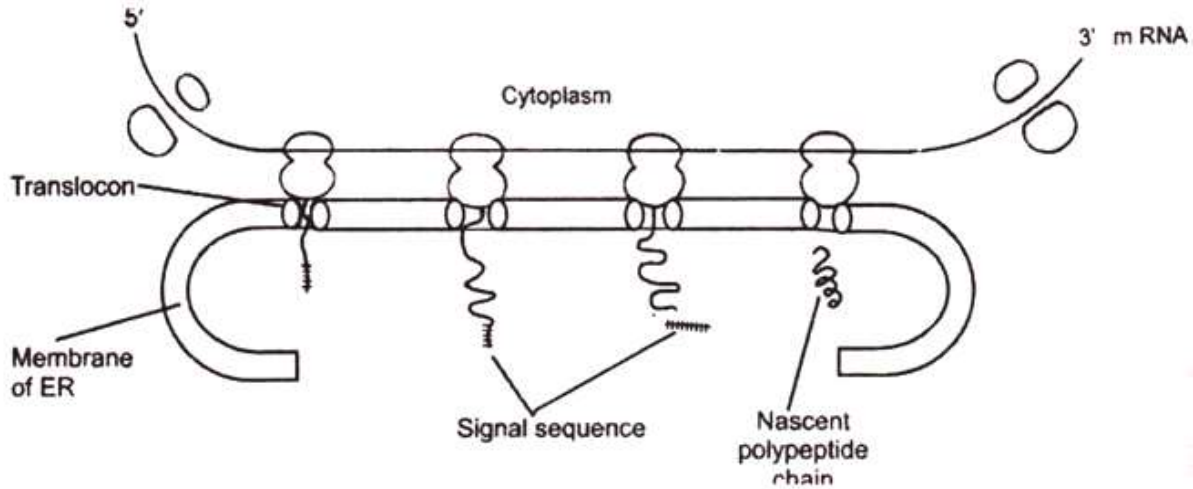


Fig. 12.12. Protein synthesis on ribosome bound on ER (RER).

Proteins synthesized by ribosomes on ER enter into the lumen of cisternae of ER from where they may enter into golgi apparatus where they are glycosylated and form secretary granules and many of them enter lysosomes.

Modification of Folding of Released Polypeptides:

DNA molecule specifies only the primary structure while folding and other modifications controlled by proteins themselves. The newly synthesized polypeptide is not always a functional protein. The newly released polypeptide may undergo various modifications. An enzyme deformylase removes the formyl group of first amino acid methionine. The cleavages of proteins are most common. Some enzymes like exo-amino-peptidases remove some amino acids either from N-terminus end or from C-terminus end or both ends. Internal amino acids may also be removed as in the case of insulin. Polyproteins are cleaved to generate individual proteins. The polypeptide chain singly or in association with other chains may fold up to form tertiary or quaternary structures. Prosthetic groups join many proteins. Some proteins assist in folding up of polypeptides. They are called chaperone proteins or chapronin proteins. Examples are Bacterial gro EL (E. coli), mitochondrial hsp60 mitonin. Various chemical common modifications of newly released proteins are glycosylation, phosphorylation, methylation, acetylation etc.

Protein Sorting or Protein Trafficking or Protein Targeting:
The proteins synthesized in the cell have to be translocated to the nucleus or other target organelles. Newly synthesized polypeptides have a signal sequence (which is a polypeptide) consisting of 13-36 amino acids. It is known as leader sequence. This signal sequence is recognized by receptors located within the membranes of the target organelles.

When proteins are synthesized on free ribosomes, the transfer takes place after the translation. When the protein synthesis takes place on ribosomes attached to the endoplasmic reticulum (Rough ER), the transfer takes place simultaneously with translation and is called co-translational transfer. The proteins which enter into the lumen of rough ER may enter into golgi apparatus, from where they may enter secretary lysosomes. The signal sequence is degraded by protease enzymes. Once all these proteins are assembled into their proper place, they provide the proper biochemical machinery, which keeps the cell feeding, locomoting, multiplying and alive.

Aminoacyl tRNA synthetases:

An aminoacyl-tRNA synthetase (aaRS or ARS), also called tRNA-ligase, is an enzyme that attaches the appropriate amino acid onto its tRNA. It does so by catalyzing the esterification of a specific cognate amino acid or its precursor to one of all its compatible cognate tRNAs to form an aminoacyl-tRNA. In humans, the 20 different types of aa-tRNA are made by the 20 different aminoacyl-tRNA synthetases, one for each amino acid of the genetic code.

This is sometimes called "charging" or "loading" the tRNA with the amino acid. Once the tRNA is charged, a ribosome can transfer the amino acid from the tRNA onto a growing peptide, according to the genetic code. Aminoacyl tRNA therefore plays an important role in RNA translation, the expression of genes to create proteins. As genetic efficiency evolved in higher organisms, 13 new domains with no obvious association with the catalytic activity of aaRSs genes have been added

Mechanism:

The synthetase first binds ATP and the corresponding amino acid (or its precursor) to form an aminoacyl-adenylate, releasing inorganic pyrophosphate (PP_i). The adenylate-aaRS complex then binds the appropriate tRNA molecule's D arm, and the amino acid is transferred from the aa-AMP to either the 2'- or the 3'-OH of the last tRNA nucleotide (A76) at the 3'-end.

The mechanism can be summarized in the following reaction series:

1. **Amino Acid + ATP → Aminoacyl-AMP + PP_i**
2. **Aminoacyl-AMP + tRNA → Aminoacyl-tRNA + AMP**

Summing the reactions, the highly exergonic overall reaction is as follows:

- **Amino Acid + tRNA + ATP → Aminoacyl-tRNA + AMP + PP_i**

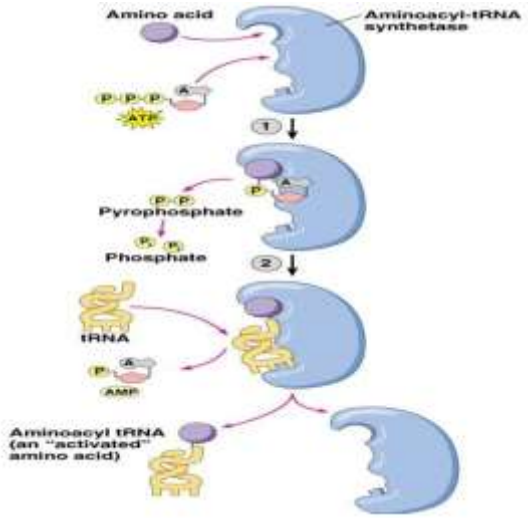


Figure: Steps involves in Amino acyl tRNA synthetase action

Some synthetases also mediate an editing reaction to ensure high fidelity of tRNA charging. If the incorrect tRNA is added (aka. the tRNA is found to be improperly charged), the aminoacyl-tRNA bond is hydrolysed. This can happen when two amino acids have different properties even if they have similar shapes—as is the case with Valine and Threonine.
The accuracy of aminoacyl-tRNA synthetase is so high that it is often paired with the word "superspecificity" when it is compared to other enzymes that are involved in metabolism. Although not all synthetases have a domain with the sole purpose of editing, they make up for it by having specific binding and activation of their affiliated amino acids. Another contribution to the accuracy of these synthetases is the ratio of concentrations of aminoacyl-tRNA synthetase and its cognate tRNA. Since tRNA synthetase improperly acylates the tRNA when the synthetase is overproduced, a limit must exist on the levels of aaRSs and tRNAs in vivo.

Classes of aminoacyl tRNA synthetase:

There are two classes of aminoacyl tRNA synthetase, each composed of ten enzymes:[3][4]

- Class I has two highly conserved sequence motifs. It aminoacylates at the 2'-OH of a terminal adenosine nucleotide on tRNA, and it is usually monomeric or dimeric (one or two subunits, respectively).
- Class II has three highly conserved sequence motifs. It aminoacylates at the 3'-OH of a terminal adenosine on tRNA, and is usually dimeric or tetrameric (two or four subunits, respectively). Although phenylalanine-tRNA synthetase is class II, it aminoacylates at the 2'-OH.

The amino acids are attached to the hydroxyl (-OH) group of the adenosine via the carboxyl (-COOH) group. Regardless of where the aminoacyl is initially attached to the nucleotide, the 2'-*O*-aminoacyl-tRNA will ultimately migrate to the 3' position via transesterification.

Structures of aminoacyl tRNA synthetase:
Both classes of aminoacyl-tRNA synthetases are multidomain proteins. In a typical scenario, an aaRS consists of a catalytic domain (where both the above reactions take place) and an

anticodon binding domain (which interacts mostly with the anticodon region of the tRNA and ensures binding of the correct tRNA to the amino acid). In addition, some aaRSs have additional RNA binding domains and editing domains[5] that cleave incorrectly paired aminoacyl-tRNA molecules.

The catalytic domains of all the aaRSs of a given class are found to be homologous to one another, whereas class I and class II aaRSs are unrelated to one another. The class I aaRSs have the ubiquitous Rossmann fold and have the parallel beta-strands architecture, whereas the class II aaRSs have a unique fold made up of antiparallel beta-strands. The alpha helical anticodon binding domain of Arginyl, Glycyl and Cysteinyl-tRNA synthetases is known as the DALR domain after characteristic conserved amino acids.

Aminoacyl-tRNA synthetases have been kinetically studied, showing that Mg2+ ions play an active catalytic role and therefore aaRs have a degree of magnesium dependence. Increasing the Mg2+ concentration leads to an increase in the equilibrium constants for the aminoacyl-tRNA synthetases' reactions. Although this trend was seen in both class I and class II synthetases, the magnesium dependence for the two classes are very distinct. Class II synthetases have two or three (more frequently three) Mg2+ ions, while class I only requires one Mg2+ ion. Beside their lack of overall sequence and structure similarity, class I and class II synthetases feature different ATP recognition mechanisms. While class I binds via interactions mediated by backbone hydrogen bonds, class II uses a pair of arginine residues to establish salt bridges to its ATP ligand. This oppositional implementation is manifested in two structural motifs, the Backbone Brackets and Arginine Tweezers, which are observable in all class I and class II structures, respectively. The high structural conservation of these motifs suggest that they must have been present since ancient times.

Accuracy during aminoacylation of tRNA:

The aminoacyl-tRNA synthetases carry out two important functions in protein synthesis: information transfer and chemical activation. The information transfer involves matching amino acids (AA) with cognate tRNA according to the rules of the genetic code. The chemical activation involves formation of a high energy ester bond between the carboxyl group of an AA and a hydroxyl of the 3′-terminal adenosine of tRNA, with an aminoacyl adenylate as intermediate.

$$\mathbf{AARS + AA + ATP \Leftrightarrow AARS \cdot AA \sim AMP + PPi}$$

$$\mathbf{AARS \cdot AA \sim AMP + tRNAAA \Leftrightarrow AARS + AA - tRNAAA + AMP}$$

Aminoacyl adenylate formation is the least accurate step in the tRNA aminoacylation pathway. Some AA [e.g., Met vs. homocysteine (Hcy); Ile vs. Val and Hcy; Leu vs. Hcy; Val vs. Cys and Thr; Ala vs. Gly; Lys vs. ornithine (Orn); or Thr vs. Ser] are so similar that AARSs misactivate them at frequencies exceeding the frequency of translational errors, forming AARS-bound noncognate aminoacyl adenylates. Noncognate adenylates are directly or indirectly destroyed by the editing function of an AARS. Editing can occur by two alternative pathways: pretransfer, by hydrolysis of the noncognate aminoacyl adenylates or post-transfer, by the hydrolysis of the mischarged tRNA. Because of the fast dissociation of aminoacyl-tRNA from AARS, post-transfer editing can contribute only a factor of ∼2 to selectivity. This may explain the more widespread use of pretransfer editing pathways by AARSs. Overall, editing

improves the AA selectivity of an AARS by a factor > 100. Consequently, nonprotein AAs Hcy or Orn are not transferred to tRNA. Some noncognate AAs are transferred to tRNA with low efficiency. For instance, IleRS promotes one misacylation of $tRNA^{Ile}$ with Val per 350,000 correct acylations with Ile. ValRS promotes one misacylation of $tRNA^{Val}$ with Ile and Thr per 5,000 and 350,000 correct acylations with Val, respectively. LysRScatalyzes one misacylation of $tRNA^{Lys}$ with Arg, Thr, Met, Leu, Ala, Cys or Ser per 1,600, 16,000, 32,000, 132,000, 265,000, 560,000 or 750,000 correct acylations with Lys, respectively. Some AARS, such as TyrRS, CysRS, ArgRS, AspRS and SerRS bind the cognate AA so much more tightly than their competitors that they do not need to edit. Overall, the accuracy of tRNA aminoacylation is greater than the accuracy of subsequent steps of translation on ribosomes.

Energetics of amino acid polymerization:

Protein synthesis itself has a higher cost than previously estimated. While transcription of the amine acid mRNA codon requires six ATP per amine acid and activation to amine acyl-tRNA requires another two ATP, there is disagreement about the total energy cost of mRNA translation. In addition to the one ATP per peptide required for capping the 5-prime end of the peptide, at least one GTP per peptide bond is required for initiation, two GTP per bond for elongation, and one GTP per peptide for termination. However, recent evidence suggests that one additional molecule of GTP is required for chain elongation (Schimmel, 1993), and hydrolysis of an additional GTP might be required during initiation and/or at the termination step as well. If hydrolysis of these additional high-energy bonds is proven correct, the net energy cost of protein synthesis alone will increase significantly from estimates made a decade ago.

In addition, there are a variety of other costs that are difficult to estimate. All of the additional sequences that are involved in, for example, synthesizing "pre-proteins" and "pre-pro-proteins" and the costs of alternate splicing are not easy to quantify. In a sense, synthesizing and then removing these unused peptide sequences is wasted energy unless some as-yet-unknown energy advantage is discovered for this process. Similarly, the cost of synthesizing nonessential amino acids that are required for protein synthesis and the costs of posttranslational modifications are not known with certainty.

Further, the folding (Hartl, 1996) and the movement of the synthesized proteins to their sites of action (Rothman and Wieland, 1996) are highly energy-dependent processes. ATP-dependent mechanisms are required for polypeptide chain folding by heat-shock protein 70 and the chaperonin families of molecular chaperones (Hartl, 1996). Translocation across the membrane of the rough endoplasmic reticulum is an energy-dependent process, as is each transport step to the cis, medial, and trans Golgi compartments (Rothman and Wieland, 1996).

n addressing the potential energy costs of regulatory proteins, one is struck by the fact that, although these proteins do not represent much in the way of mass compared with structural proteins, their turnover rates are very high. Therefore, they may represent a significant energy drain. It is now known, for example, that an immense number of processes are controlled by reversible enzymatic phosphorylation/dephosphorylation reactions. This central mechanism of regulatory control occurs in every cell at an unimaginable number of times per minute. What the actual net energy cost of these regulatory events is to the whole body is only speculative, but likely significant.

Signal transduction processes, including those mediated by the more than 100 known members of the protein kinase family, and the energy-dependent costs of the second messenger families, for example the phosphatidyl and inositol kinases, are additional energy costs of regulatory protein metabolism. Furthermore, the energy costs of posttranslational modifications such as protein glycosylation are additional. The addition of each nucleotide sugar costs the hydrolysis of one uridine triphosphate bond. Approximately 10 percent of proteins are glycosylated. Each protein averages between 2 and 5 sugar chains, and each sugar chain averages about 12 sugars. Precisely how to tally this cost in the overall sum of the total daily energy cost of protein turnover, is not known, but the cost is potentially large.

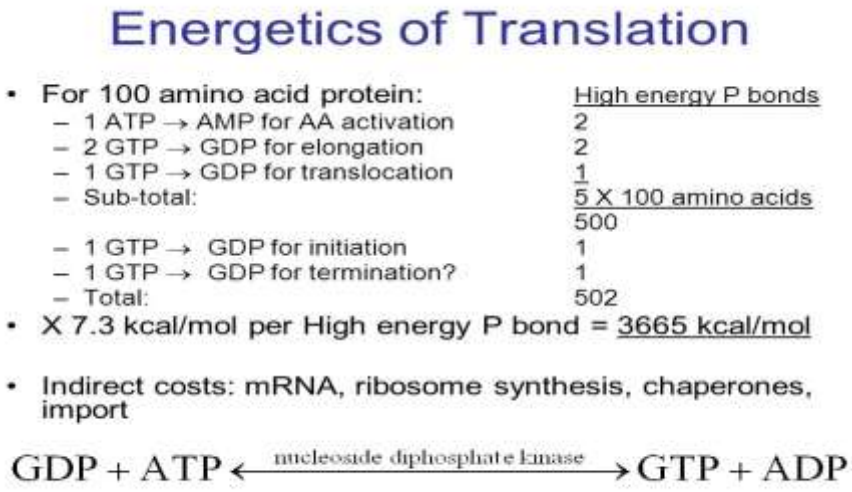


Figure: Energetics of Translation

Inhibitors of Protein Synthesis:
There are many chemicals, both synthetic as well as those obtained from different sources like fungi, which bind to the components of translation machinery and arrest the translation process. Most of them are antibacterial agents or antibiotics that act exclusively on bacteria and are thus powerful tools in the hands of man to combat various infectious diseases. Most of antibiotics are inhibitors of translation machinery.

a. Puramycin: It binds at "A" site on ribosome. This causes pre-mature termination of polypeptide chain.
b. Kirromycin: It inhibits the elongation factor EF-Tu.
c. Fusidic acid: It inhibits the elongation factor EF-G.
d. Tetracycline: It attacks "A" site on ribosome and prevents the binding of aminoacyl- tRNA.
e. Chloramphenicol: It blocks the peptidyl transfer reaction.
f. Erythromycin: It binds the polypeptide exit channel of ribosome, therefore blocks the exit of growing polypeptide chain, thus stops the translation process.
g. Streptomycin and Neomycin: These inhibit the binding of $tRNA^{fMet}$ to the "P" site.

Inhibitors in Eukaryotes:
Diphtheria toxin is a toxin produced by *Corynebacterium diphtheriae*. This causes modification of eukaryotic elongation factor.

Probable Questions:

1. Describe structure of nuclear envelop with diagram.
2. Write the functions of nuclear envelope?
3. How nuclear membrane assembled and disassembled?
4. Describe structure of nuclear pore complex.
5. How nuclear export has occurred?
6. How nuclear import has occurred?
7. How nuclear transport is regulated?
8. What are the main components of translation?
9. Write down the structure of tRNA.
10. Discuss the role of ribosome in protein translation.
11. Describe the initiation process of translation.
12. How termination occurs in protein translation
13. Describe the elongation phase of translation.
14. What is polysome complex?
15. How amino acyl tRNA works?
16. describe different inhibitors of protein translation.
17. describe different classes of aminoacyl tRNA synthetase.
18. Describe energetics of protein translation.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

UNIT-IV

Basic recombinant DNA techniques, cutting and joining DNA molecules, restriction modification systems, various enzymes used in recombinant DNA technology, restriction maps and mapping techniques; DNA foot printing, methyl interference assay. Polymerase chain reaction– methods and applications

Objective: In this unit we will discuss about different types of Restriction endonuclease with their property and mechanism of actions. Also we will discuss about restriction mapping, DNA fingerprinting and DNA foot printing methods. We will also discuss about PCR and methyl interference assay.

Restriction Endonuclease:

Restriction endonuclease enzymes occur naturally in bacteria as a chemical weapon against the invading viruses. They cut both strands of DNA when certain foreign nucleotides are introduced in the cell. Endonucleases break strands of DNA at internal positions in random manner.

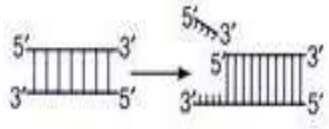
These are also known as molecular scissors, used for cutting of DNA. The cutting of DNA at specific locations became possible with the discovery of molecular scissors, i.e., restriction enzymes. In the year 1963, the two enzymes responsible for restricting the growth of bacteriophage in E. coli were isolated. One of these added methyl groups to DNA, while the other cuts the DNA. Later was termed as restriction endonucleases.

The first restriction endonuclease was isolated by Smith Wilcox and Kelley in 1968 was Hind II. It was found that it always cuts DNA molecules at a particular point by recognising a specific sequence of six base pairs known as recognition sequence for Hind II. Today, more than 900 restriction enzymes have been isolated from over 230 bacterial strains each of which recognise different recognition sequences.

Differences between Exonucleases and Endonucleases

Exonucleases:

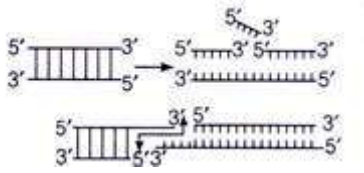
1. These nucleases cleave base pairs of DNA at their terminal ends
2. They act on single – strand of DNA or gaps in double –stranded DNA. They do not cut RNA



Endonucleases:

1. They cleave DNA at any Pont except the terminal ends

2. They cleave one strand (figure below) or both strands (figure below) of double – stranded DNA. They may cut RNA



Each restriction endonuclease recognizes a specific palindromic nucleotide sequences in the DNA. Palindrome in the DNA is a groups of letters that forms the same words when read both forward and backward. For example, the following sequences read the same on the two strands in 5′ → 3′ direction as well as 3′ → 5′ direction.

5′ —— GAATTC ——3′
3′ —— CTTAAG ——5′

Types of Restriction Enzymes:

1. Restriction enzyme Type I:

These enzymes interact with an unmodified recognition sequence in double-stranded DNA and then attach to long DNA molecule. After travelling for distance between 1000 to 5000 nucleotides the enzymes cleaves only one strand of the DNA at an apparently random site, and creates a gap of about 75 nucleotides in length.

Acid soluble oligonucleotides removed from the gap are released. A second enzyme molecule is needed to cleave the remaining strand of DNA. The cofactors for the enzyme are Mg^{2+} ions, ATP and S-adenosyl- methionine. This kind of enzyme is not useful for genetic engineering, because its cleavage sites are non-specific.

Type I restriction enzyme can simultaneously hold two different sites on DNA creating a loop in nucleic acid. This enzyme consists of three types of subunits. The Eco K enzyme, for example, has the structure R_2M_2S. The R subunit is responsible for restriction and the M subunit for methylation. The binding of enzyme to DNA may be succeeded by either restriction or modification and this property is characterized by S subunit.

2. Restriction enzyme Type II:

These enzymes recognise a particular target sequence in a double-stranded DNA molecule. They cleave the polynucleotide chain within or near that sequence to give rise to distinct DNA fragments of defined length and sequence. They require Mg^{2+} ions for the action (i.e., restriction). Type II enzymes are used for gene manipulation studies.

3. Restriction enzyme Type III:

These enzymes cleave double-stranded DNA at well-defined sites. They require ATP, Mg^{2+} ions and have very partial requirement for S-adenosyl-methionine for restriction. They have intermediate properties between Type I and Type II REs.

Naming of Restriction Endonuclease Enzymes:

About 350 types of restriction endonucleases have been isolated from more than 200 bacterial strains. Large number of these enzymes require a system of uniform nomenclature. A system based on the proposals of Smith and Nathans (1973) has been followed for the most part.

Table 55.2. Characteristics of restriction endonuclease enzymes.

Characteristics	Type I	Type II	Type III
1. Restriction and modification activities	Single multifunctional enzyme	Separate endonuclease and methylase	Separate enzymes with a subunit is common
2. Protein structure of enzyme	Three different subunits	Simple	Two different subunits
3. Requirements for restriction	ATP, Mg^{2+} S–adenosyl-Mg^{2+} methionine		ATP, Mg^{2+} (S-adenosyl methionine)
4. Sequence of host specificity sites	*Eco* B: TGAN*$_8$TGGT *Eco* K: AA N*$_6$ GTGC	Usually rotational symmetry	*Eco* P1: AGACC *Eco* P15: CAGCAG
5. Cleavage sites	Possibly random at least 1000 bp from host specificity site	At or near host specificity site	24-26 bp to 3' of host specificity site
6. Enzymatic turnover	No	Yes	Yes
7. DNA translocation	Yes	No	No
8. Site of methylation	Host specificity site	Host specificity site	Host specificity site

Naming exercise of RE enzymes is based on following rules:

1. Each RE enzyme is named by a three-letter code.
2. The first letter of this code is derived from the first epithet (first letter of name) of the genus name. It is printed in italics.
3. The second and third letters are from the first two letters of its species name. They are also printed in italics.
4. This is followed by the strain number. If a particular strain has more than one restriction enzyme, these will be identified by Roman numerals as I, II, III, etc.

For example, the enzyme Eco RI was isolated from the bacterium Escherichia (E) coli (co) strain RY13 (R) and it was the first endonuclease (I). R also indicates antibiotic resistant plasmid of the bacterium. Likewise, Hind II from Haemophilus influenzae strain Rd and Bgl I from Bacillus *globigii*. A few restriction endonuclease enzymes and their sources are given in Table 55.3.

Table 55.3. A few restriction endonuclease enzymes, their sources and recognition sites.

Name of the restriction endonuclease enzyme	Source (Microorganisms)	Recognition sequence and cleavage site
1. *Aat* II	*Acetobacter aceti*	GACGT ↓ C
2. *Bcl* I	*Bacillus Caldoyticus*	T ↓ GATCA
3. *Cvn* I	*Chromatium vinosum*	CC ↓ TNAGG
4. *Eco* RI	*Escherichia coli* RY13	G ↓ AATTC
5. *Eco* RII	*Escherichia coli R*245	↓ CCTGG
6. *Hind* II	*Haemophilus influenzae* Rd	GTP_y ↓ PuAC*
7. *Hind* III	*Haemophilus influenzae* Rd	A ↓ AGCTT
8. *Kpn* I	*Klebsiella pneumoniae* OK	GGTAC ↓ C
9. *Nop* I	*Nocardia opaca*	G ↓ TC GAC
10. *Nsp* B II	*Nostoc*	C(A/C)G ↓ C(T/G)G
11. *Sfa* I	*Streptococcus faecalis*	GG ↓ CC
12. *Sal* PP	*Streptomyces albus*	GTGCA ↓ G
13. *Sal* I	*Streptomyces alubs* G	G ↓ TC GAC
14. *Xor* II	*Xanthomonas oryzae*	CGAT ↓ CG
15. *Xba* I	*Xanthomonas badrii*	T ↓ CTAGA

*Pu = purine Py = pyrimidine

Target Sites of Restriction Endonuclease Enzymes:

A restriction endonuclease enzyme of type H recognises a specific recognition site (base sequence) on the DNA and makes a cut at this site only. These target sites are 4 to 6 nucleotides long (Fig. 55.3). They exhibit palindromic symmetry, i.e., nucleotide pair sequences are same reading forward or backward from a central axis of symmetry, like the nonsense phrase-AND 'MADAM DNA'.

The term palindromic has also been applied to sequences such as:

5′-AGCCGA—

3′-TCGGCT— both of which are palindromic strands.

X-ray crystallography of RE enzyme-DNA complex indicate that endonuclease acts as a dimer of identical subunits and that the palindromic nature of target sequence reflects the two fold rotational symmetry of the dimeric protein.

Nature of Cut Ends:

Two types of cut ends of DNA, namely blunt or flush ends and sticky or cohesive ends, are produced by the restriction endonuclease s. The nature of these cut ends generated by the REs are very important in designing the gene cloning experiments.

1. Blunt cut ends:

In case of the blunt cut end, the enzyme (e.g., Haelll, Smal) makes a simple double-stranded cut in the middle of the recognition sequence. Thus the blunt ends or flush ends are formed. The RE Hae III makes a cut in the 5′-GGCC-3′ target site as shown in Fig. 55.3.

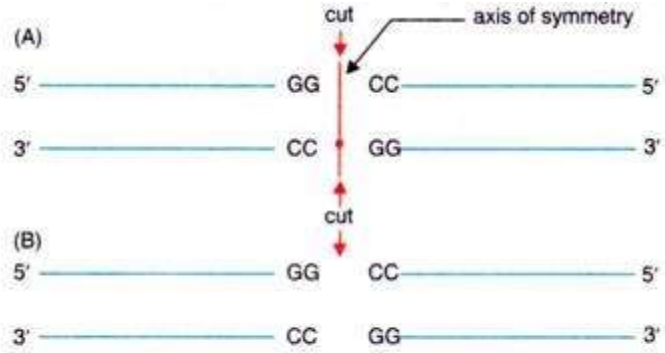


Fig. 55.3. A-The target site for restriction endonuclease *Hae* III. Arrows indicate the sites where the cuts will be affected. B– The target site after the cut. As a result of this the ends of cut DNA are blunt, that is protruding unpaired bases.

The utility of generation of blunt end cuts during the joining of DNA fragments is that any pair of ends may be joined together irrespective of sequence. This is especially useful for those researchers who are interested to join two defined sequences without introducing any additional material between them. Table 55.4 shows certain blunt-end restriction sites.

Table 55.4. Examples of certain blunt end restriction sites and restriction endonucleases.

Enzyme	Recognition site	Cleavage product
1. *Hae* III	–GG ↓ CC –	–GG CC–
2. *Hae* III	–CC ↑ GG –	–CC GG–
3. *Sma* I	–CCC ↓ GGG –	–CCC GGG–
4. *Sma* I	–GGG ↑ CCC –	–GGG CCC–

2. Sticky-or Cohesive ends:

Many restriction enzymes (e.g., Eco RI, Bam HI, and Hind III) make staggered, single-stranded cuts, producing short single-stranded projections at each end of the cleaved DNA, called sticky ends. Since the restriction sites are symmetrical, so that both strands have the same sequence when read in the 5′ to 3′ direction. Thus, such staggered cuts will generate identical single-stranded projections on the either site of the cut (Fig. 55.4).

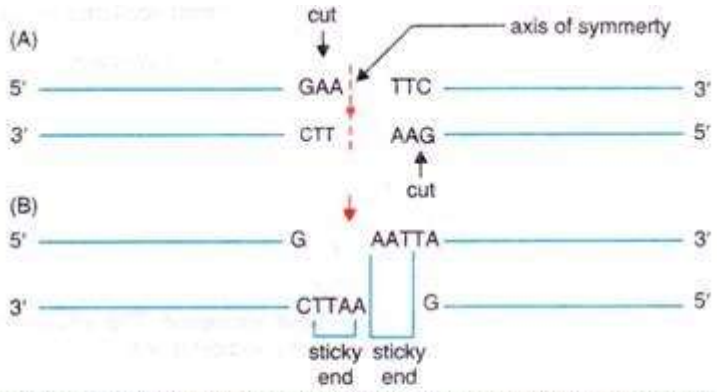


Fig. 55.4. A–Diagram showing the recognition site of the restriction endonuclease *Eco* RI. This enzyme makes a cut in the sugar-phosphate backbone between the nucleotides with bases guanine and adenine on both the polynucleotides with bases guanine and adenine on both polynucleotide strands (shown by arrows). B–This leads to the formation of DNA fragments with sticky ends.

Table 55.5. Examples of certain sticky– or cohesive-end restriction enzymes and sites.

Enzyme	Recognition site	Cleavage product
1. *Eco* RI	–G ↓ AATTC–	–G AATTC–
2. *Eco* RI	–CTTAA ↑ G–	–CTTAA G–
3. *Hind* III	–A ↓ AGCTT–	–A AGCTT–
4. *Hind* III	–TTCG A ↑ A–	–TTCGA A–

These ends are not only identical, but complementary, and will base pair with each other; they are therefore, known as cohesive or sticky ends. Because of specificity of restriction enzymes, every copy of a given DNA molecule will give the same set of fragments when cleaved with a particular enzyme. Different DNA molecules will in general, give different sets of fragments when treated with the same enzyme. The table 55.5 shows sticky or cohesive-end restriction enzymes and sites:

Host Controlled Restriction and Modification:

Certain strains of bacteria are immune to bacteriophages. This phenomenon is called host controlled restriction. This restriction is due to these restriction endonuclease enzymes (e.g., Eco RI) which could recognise and split specific loci in the foreign DNA. Thus these enzymes prevent or restrict the survival of foreign DNA in the host. This is analogous to an immune system.

All restriction sites in host chromosome of a bacterium are protected from its own restriction endonuclease enzyme due to a modification system. This system helps in preventing suicidal self-degradation.

Such modification occurs by methylation of specific bases in the recognition sequence of the endonuclease. The enzymes involved in such modification are called methyltransferases. These enzymes methylate adenine (i.e., adds a methyl group to the base) in the N6 position and cytosine either in N5 or W position and produce 6 methyl adenine and 5 or 4 methyl cytosine respectively. Unmodified foreign DNA entering the cell is degraded by the host restriction

system. As both the enzymes, i.e., methyltransferases and endonucleases recognise the restriction site, they are together called as restriction and modification system.

Star Activity:

Various REs show , the star activity when they exhibit relaxation in specificity of sequence under non-optimal conditions. In such condition, endonuclease enzymes even recognise other alternative base instead of a specific base. The following factors are known to alter the DNA recognition sequence for several to alter the DNA recognition sequence for several endonuclease enzymes: non-ideal strength buffers, high glycerol concentration (more than 5% v\v) and high enzyme concentration.

Isoschizomers:

Isoschizomers are restriction endonuclease enzymes which are isolated from different organisms but recognize identical base sequences in the DNA. For example, Asp 718 and Kpn I have identical recognition sites-

Source of Asp718 is Achromobacter species 718; source of Kpn I is Klebsiella pneumoniae OK 8. Some pairs of isochizomers cut their target at different places (e.g., Sma I, Xma I).

Use of Restriction Endonuclease Enzymes in Genetic Engineering:

In gene cloning experiments, DNA molecules have to be cut in a very precise and reproducible manner. Restriction endonuclease enzymes play an important role in cutting the desired gene as well as cleaving the vector.

1. Cutting the gene:

The required DNA fragment from a large DNA molecule should be cleaved in a precise manner for further genetic manipulations. A particular restriction endonuclease enzyme can recognize and bind to specific base sequence of the DNA and then will cleave it. It is highly reproducible and can be programmed according to DNA sequences of required gene and particular endonuclease enzymes identifying and cleaving it.

2. Cutting the vectors:

The function of a vector DNA molecule is to carry a gene of interest to a second organism where it can express it (i.e., can produce a gene specific product). During this technique the DNA to be cloned is integrated with the plasmid. Hence each vector molecule should be cleaved with same restriction site at a single position to open the circular form so that the new DNA fragment can be inserted at these complementary sites.

If foreign DNA is introduced into E. coli host, it may be attacked by restriction endonucleases active in a host cell. Because restriction phenomenon provides a natural defence against invasion by foreign DNA, it is usual to employ a K restriction deficient E. coli K12 strain as a host in transformation with newly created recombinant DNA molecules. This will eliminate the chance that the incoming sequence will be restricted.

Restriction Enzymes as Tools:

Recognition sequences typically are only four to twelve nucleotides long. Because there are only so many ways to arrange the four nucleotides—A,C,G and T–into a four or eight or twelve nucleotide sequence, recognition sequences tend to "crop up" by chance in any long sequence. Furthermore, restriction enzymes specific to hundreds of distinct sequences have been identified and synthesized for sale to laboratories.

As a result, potential "restriction sites" appear in almost any gene on chromosome. Meanwhile, the sequences of some artificial plasmids include a "linker" that contains dozens of restriction enzyme recognition sequences within a very short segment of DNA. So no matter the context in which a gene naturally appears, there is probably a pair of restriction enzymes that can cut it out, and which will produce ends that enable the gene to be spliced into a "plasmid". Another use of restriction enzymes can be to find specific SNPs. If a restriction enzyme can be found such that it cuts only one possible allele of a section of DNA (that is, the alternate nucleotide of the SNP causes the restriction site to no longer exist within the section of DNA), this restriction enzyme can be used to genotype the sample without completely sequencing it. The sample is first run in a restriction digest to cut the DNA, and then gel electrophoresis is performed on this digest.

If the sample is homozygous for the common allele, the result will be two bands of DNA, because the cut will have occurred at the restriction site. If the sample is homozygous for the rarer allele, the sample will show only one band, because it will not have been cut. If the sample is heterozygous at that SNP, there will be three bands of DNA. This is an example of restriction mapping.

Other important enzymes which are used in Recombinant DNA Technology:

1. Alkaline Phosphatases:

The enzyme alkaline phosphatase (AP) catalyses the removal of the 5′-terminal phosphate residues from nucleic acids (RNA, DNA and ribo- and deoxyribonucleotide triphosphates). This enzyme is isolated from bacteria (BAP) or calf intestine (CAP). This enzyme is a dimeric glycoprotein with a molecular weight 14,000. It is made up of two identical or similar subunits each with a molecular weight of 6900. It is a zinc-containing enzyme with four atoms of Zn^{2+} per molecule.

Uses of Alkaline Phosphatase Enzyme:

1. Linearized cloning vectors can be prevented from recircularizing by dephosphorylation with alkaline phosphatase enzyme.
2. The free 5′-OH can be phosphorylated with polynucleotide kinase and Υ^{-32P} ATP to produce 32P end labelled nucleic acid.
3. AP enzyme is used for mapping and DNA fingerprinting studies.

2. Taq Polymerase:

Taq polymerases are DNA dependent DNA polymerase from Thermus aquaticus, primarily used for synthesis of longer stretches of DNA. Thermus aquaticus is the source for this enzyme which is an extreme thermophile, living in hot springs.

Due to its thermophillic habitats, it shows extreme resistant to high temperature, which exhibits peak of activity at temperature optimum of 72°C. Taq polymerase consists of single polypeptide chain with a molecular weight of 90,000 daltons. This enzyme is particularly useful when reactions need to take place at high temperature, denaturation of high G + C content into DNA. Thermostable property of this enzyme is significant in the polymerase chain reaction, where it is used to extend primers in the process of repeated rounds of heating, cooling and complete the synthesis of new DNA strand.

3. Nucleases:

Nucleases are DNases and RNases. DNases digest both the strands of DNA. It can hydrolyse each DNA strand independently in presence of Mg^{+} ion. DNases are used in the purification of DNA by eliminating contaminated DNA. It is also widely used in foot printing and nick translation.

RNases cleaves phosphodiester bond between the two adjacent nucleotides. RNase cleaves the bond next to uracil and guanine. Aspergillus and bovine pancreas are the chief source for RNase. The RNase H is widely employed in cDNA preparation where it removes mRNA from RNA-DNA hybrid. Removal of polyA tail requires RNase.

4. Reverse Transcriptase Enzyme:

The enzyme reverse transcriptase is isolated from avian myeloblastos virus (AMV). It is an RNA-dependent DNA polymerase. The enzyme requires DNA primer complementary to the RNA template, as well as presence of Mg^{2+} or Mn^{2+} for initiation of transcription. Reverse transcriptase mediates the conversion of genetic information present in single- stranded molecule of RNA into a double-stranded molecule of DNA.

Until recently, it was known that the genetic information's of DNA pass to protein through mRNA. During 1960s, Temin and coworkers postulated that in certain cancer causing animal viruses which contain RNA as genetic material, transcription of cancerous genes (on RNA into DNA) takes places most probably by DNA polymerase directed by viral RNA. Then DNA is used as template for synthesis of many copies of viral RNA in a cell. In 1970, S. Mizutani, H.M. Temin and D. Baltimore discovered that information can pass back from RNA to DNA. They found that retroviruses (possessing RNA) contain RNA dependent DNA polymerase which IS also called reverse transcriptase. This process produces single- stranded DNA which in turn functions as template for complementary chain of DNA. Reverse transcriptase enzyme has two subunits. The enzymatically active forms of the purified enzyme are α, β and αβ. The molecular weight of the α-subunit is 68000 and that of β-subunit is 92,000. The mature α-β form is the most active form of AMV reverse transcriptase enzyme. It has several enzymatic roles such as RNA-directed DNA polymerase action, DNA dependent RNA polymerase activity and RNase-H activity. The α- subunit of reverse transcriptase contains the polymerase activity. It also has the RNase- H activity during which degradation of RNA in DNA: RNA hybrids takes place. Such a sort of exonucleolytic activity of RNase-H enzyme can proceed either from the 5′- or 3′- terminus.

Uses of Reverse Transcriptase Enzyme:

a. The in vitro synthesis of cDNA from mRNA and other RNA molecule using reverse transcriptase enzyme has become a very important technique in the field of molecular biology.

b. DNA-dependent DNA polymerase activity of reverse transcriptase enzyme is responsible for second-strand formation in cDNA synthesis. Such a polymerising activity of reverse transcriptase is inhibited by the addition of actinomycin-D.

c. The reverse transcriptase enzyme mediates the conversion of genetic information present in single-stranded molecule of RNA into a double-stranded molecule of DNA.

5. DNA Ligating Enzyme (Ligases):

Ligases acts as a key player in genetic engineering experiment for its role as molecular suture, where it facilitates joining of DNA fragments. The main source of this enzyme is the T4 phage virus. The two DNA pieces are efficiently joined by DNA ligase.

The joining of DNA fragments requires ATP for T4 DNA ligase or NAD^+ in the case of E. coli ligase. Joining of DNA fragments is accomplished by forming a covalent bond between the 5′ phosphoryl of one strand and 3′ hydroxyl of the adjacent strand. Thus, it catalyses the end to end joining of DNA duplex at the base paired end. During sealing process, two phosphodiester bonds are formed by T4 DNA ligase. The stability of joined fragments is due to the formation of 3′ to 5′ phosphotodiester linkage between cohesive ends. The blunt ends produced by certain restriction enzymes may hinder the sealing process (Fig. 13.1). Hence, it is estimated to use ligase at high strength to accomplish sealing process.

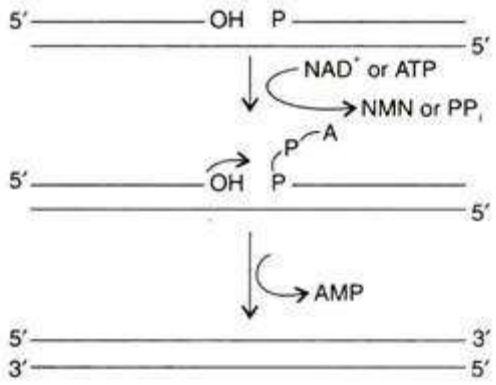


Fig. 13.1 Mechanism of DNA ligation

6. DNA Polymerase and the Klenow Fragment:

The DNA polymerase that is generally utilized is either the DNA Pol I from E. coli or the T4 DNA polymerase encoded by the phage gene. The E. coli enzyme is basically a proof-reading and repairing enzyme. It is composed of 3 subunits each with a specific activity. They are: 5′-3′ polymerase, 3′-5′ exonuclease and 5′-3′ exonuclease.

The enzyme is useful for synthesizing short length of a DNA strand, especially by the nick translation method. The 5-3′ exonuclease activity may be deleted, this edited enzyme is referred to as the Klenow fragment. The T4 DNA Pol possesses, like the klenow fragment, only the polymerase and proofreading (3′-5′ exonuclease) functions.

7. Phosphonucleotide Kinase Enzyme:

The enzyme phosphonucleotide kinase catalyses the transfer of the terminal phosphate group of ATP to the 5′-hydroxylated terminal of DNA or RNA. This enzyme is frequently used to end- label the nucleic acids with ^{32}P (i.e., it adds the phosphate back to 5′-termini of DNA).

This can be accomplished by any method among following:

a. Forward reaction:

Transfer of labelled ϒ-phosphate form (ϒ-^{32}P)-ATP to the free 5′-hydroxyl group of substrate-

5′ – OH – DNA + [^{-32}P] ATP5′^{32} → PO – DNA + ADP

Substrate lacking a free 5′-hydroxyl requires prior dephosphorylation by alkaline phosphatase.

b. Exchange reaction:

In the initial step, the terminal 5′-phosphate is transferred from substrate to ADP present in the reaction mixture. Then, the labelled ϒ-phosphate from [ϒ-^{32}P]-ATP is transferred to free hydroxyl group of substrate.

$$5' – PO – DNA + ADP \rightarrow 5'HO – DNA + ATP$$

$$5' – HO – DNA + [\Upsilon^{-32} P] – ATP \rightarrow 5'^{32} PO – DNA + ATP$$

Uses of Polynucleotide Kinase Enzyme:

The enzyme polynucleotide kinase is used to label 5′-termini of DNA and RNA with [ϒ-^{32}P]-ATP by phosphorylation of 5′-hydroxyl groups or by the exchange reaction. This 5′-terminal labelling is used in mapping of restriction sites, DNA or RNA fingerprinting, hybridization studies and sequence analysis of DNA.

8. S1 Nuclease Enzyme:

The S1 nuclease enzyme is single- strand specific endonuclease which cleaves DNA to release 5′-mono and 5′-oligonucleotides. Normally, double- stranded DNA, double- stranded RNA and DNA-RNA hybrids are resistant to action of S1 nuclease enzyme.

However, very large amounts of S1 nuclease enzyme can completely hydrolyze double-stranded nucleic acids. The enzyme hydrolyzes single stranded regions in duplex DNA such as loops and gaps. S1 nuclease enzyme can also cleave single stranded areas of super helical DNA at torsional stress points where DNA may be unpaired or weakly hydrogen bonded. Once the super-helical DNA is nicked, S1 nuclease enzyme can cleave the second strand near the nick to generate linear DNA. S1 nuclease enzyme is a monomeric protein with 3800 Dalton molecular weight. It requires Zn^{2+} for its activity and is relatively stable against denaturing reagents such as urea, SDS and formamide. The optimum pH requirement lies between 4 to 4.5.

Uses of S1 Nuclease Enzyme:

1. S1 nuclease enzyme is used to analyse DNA-RNA hybrid structures to map transcripts.

2. It can be used to remove singles stranded tails from DNA fragments to produce blunt ends.

3. Hair pin loop structures formed during synthesis of double-stranded cDNA is digested by this enzyme.

4. S1 nuclease enzyme is also used for DNA mapping, called SI nuclease mapping Turner.

9. Ribonuclease:

Generally RNase A and RNase T1 enzymes are used in genetic engineering techniques. Both enzymes cleave the phosphodiester bond between adjacent ribonucleotides. RNase A cleaves next to uracil (U) and cytosine (C) in such a way that phosphate remains with these pyrimidines. The nucleotide present on the other side of phosphate is dephosphorylated. RNase A enzyme is isolated from the bovine pancreas.

RNase T1 cleaves specifically next to guanine. The phosphate group at the 3′ end of the nucleotide remains with the cut end. This enzyme is isolated from *Aspergillus oryzae.*

10. Ribonuclease H (RNase H):

The enzyme RNase H is an endoribonuclease that degrades the RNA portion of the RNA- DNA hybrids. RNase H enzyme cuts the RNA into short fragments.

Applications of RNase H:

a. RNase H is the key enzyme in the cDNA cloning technique. In this case, it is used to remove the mRNA from the RNA-DNA hybrid.

b. RNase H enzyme is used to detect the presence of RNA-DNA hybrid.

c. RNase H enzyme is used to remove poly (A) tails on mRNA.

11. Deoxyribonuclease I (Dnase I):

The enzyme DNase I is an endonuclease enzyme which digests either single or double-stranded DNA, producing a mixture of mononucleotides and oligonucleotides. DNase I hydrolyses each strand of double-stranded DNA independently and at random. Addition of Mg^{2+} to reaction mixture ensures random cleavage while addition of Mn^{2+} gives cleavage nearly at the same place on both strands. DNase enzyme is obtained mostly from bovine pancreas.

Uses of DNase I Enzyme:

DNase 1 enzyme is useful for a variety of applications including nick translation, DNA foot printing, bisulphite mediated mutagenesis and RNA purification.

12. Terminal Deoxynucleotidyl Transferase Enzyme:

The enzyme deoxynucleotidyl transferase catalyses the repetitive addition of monodeoxynucleotide units from a deoxynucleoside triphosphate to the terminal 3′-hydroxyl group of a DNA molecule. This enzyme has a molecular weight of 32000 and consists of two subunits each with a molecular weight of 26500 and 8000. This enzyme is isolated from calf thymus.

Uses of Terminal Transferase Enzyme:

1. The enzyme terminal transferase is used to add homopolymer tails of DNA fragments. Using a technique called homopolymer tailing, sticky ends can be built up on blunt-ended DNA

molecules. For examples, one preparation of DNA could be treated with the enzyme terminal transferase in the presence of dATP, resulting in the addition of a poly (dA) chain to each DNA strand. There is another preparation of DNA which provides 3 tails of poly (T) using same enzyme with TTP. When both types of DNA preparations DNA fragments with poly A tails and DNA fragments with poly T tails, are mixed, there takes place base pairing between complementary sticky ends, which could then be ligated. One advantage of this method is that ligation does not take place between fragments from the same DNA preparation.

2. Terminal transferase enzyme is used for 3′-end labelling of DNA fragments
3. Terminal transferase enzyme is also used for the addition of single nucleotides to the 3- end of DNA for in vitro mutagenesis.

Restriction Mapping:

A description of restriction endonuclease cleavage sites within a piece of DNA is referred to as a restriction map. Such a map is usually generated as the first step in characterizing an unknown DNA, and a prerequisite to manipulating it for other purposes. Restriction enzymes that cleave DNA infrequently (e.g. those with 6 bp recognition sites) are relatively inexpensive are used to produce at a map (Chakraborty, Pandey, et.al., 2006). Restriction sites are specific recognition sites where enzymes known as endonucleases cleave the DNA. e.g. EcoRI cuts at GAATTC (Gale, 2003). When discovered in archaea and bacteria, these enzymes were part of the defence mechanism of such organisms, limiting the foreign DNAs to act upon the cell. Theses enzymes will defend cells by digesting invading DNA into small, non-functional pieces. Thus this is where the name “restriction enzyme” comes from; the function of the enzyme, i.e. the ability of the enzyme to restricting access to the cell (Carroll, Griffiths, et.al., 2008). Restriction maps show the relative location of a selection of restriction sites along linear or circular DNA.
Restriction mapping involves a series of restriction enzymes digesting the DNA and then separating the resultant fragments by agarose gel electrophoresis. The patterns of fragments that are produced by restriction enzyme digestion determine the distance between restriction enzyme sites; this is how information about the structure of an unknown piece of DNA can be obtained (Champness & Snyder, 2007).

Techniques of Restriction Mapping.
There are several methods for restriction mapping; the most straightforward being the digesting of samples of the plasmid with a set of individual and pairs of those enzymes; these digests are then “run out” on an agarose gel to determine sizes of the fragments generated.
Consider to illustrate these ideas, a plasmid that contains a 3000 base pair (bp) fragment of unknown DNA. Immediately flanking the unknown DNA within the vector are unique recognition sites for the enzymes Kpn I and BamH I. Then, consider digestions with Kpn I and BamH I separately. In essence, single digests are used to determine which fragments are in the unknown DNA, and double digests to order and orient the fragments correctly (Chakraborty, Pandey, et.al., 2006). If a DNA fragment is labelled with a radioisotope on one end only, this can directly reveal where the cleavage sites are located as by partially digesting the fragment with restriction enzymes, labelled fragments are generated (Chakraborty, Pandey, et.al., 2006).

If the sequence is known, any number of computer programmes can be used to build up a map. It is simply a matter of feeding the sequence into the programme which will then search the sequence for dozens of restriction enzyme recognition sites and build you a map (Chakraborty, Pandey, et.al., 2006).

Uses and Applications of Restriction Mapping:

Restriction map information is important for many techniques used to manipulate DNA; one application being the cutting large pieces of DNA into smaller fragments for allowing it to be sequenced. Another application is to use restriction mapping to compare DNA fragments without having any information of their nucleotide sequence (Gale, 2003).

Restriction mapping has contributed immensely towards our knowledge of vectors and plasmids (OUP, 1995). It has also contributed heavily to our ability to genetically engineer organisms and recombinant DNA technology where an organism's genes are manipulated indirectly; examples of this include the generation of synthetic human insulin using modified bacteria and the production of erythropoietin in hamster ovary cells, amongst many more (Banting, 1929).

Industries like medicine, agriculture etc. also use this technique for the production of several medically useful substances like the hepatitis-B vaccine, human interferon and human growth hormone. Identifying the sequences with restriction mapping has allowed for plants to produce their own pesticides ant to perform nitrogen fixation by genetically adjusting the plant species. Bacteria capable of biodegrading oil have been produced using this technique for the use in oil-spill clean ups. The technique of restriction mapping has its applications in the field of gene knock out experiments in mice as well as identification of gene before introduction into a foreign organism to make transgenic pigs and cats. Similarly, we have been able to express several medicinal proteins in bacterial systems using restriction techniques; the most famous examples are insulin(Banting 1929).

Is restriction mapping still useful?

The process of restriction mapping is simple and easy. It can be carried out in 1-2 days. The advancements in the field of computing have enabled automated softwares to virtually analyze the sequence by identifying the restriction sites. Restriction mapping is a helpful tool for experiments where sequencing can be out of budget or not necessary. It can be used to determine whether a gene has been cloned into the plasmid. It is a much better technique for relatively short segments of DNA.

Technologies That Succeeded Restriction Mapping.

One major method that has replaced restriction mapping is the High-Throughput Sequencing and Genotyping, which is to facilitate the unraveling of genetic information across the large and diverse collection of animals, plants and microbes. This has been very useful in cases where DNA does not contain any known restriction sites, or DNA, which contains sites for enzymes, which are not commercially available. It is also advisable to send the sample for sequencing when the sample is very small (Mitchelson, 2007).

Restriction fragment length polymorphisms (RFLP) are variations in DNA fragment-banding patterns (from different individuals of a species) of electrophoresed restriction digests of DNA (Appa Rao, Mohan, et.al., 1994).

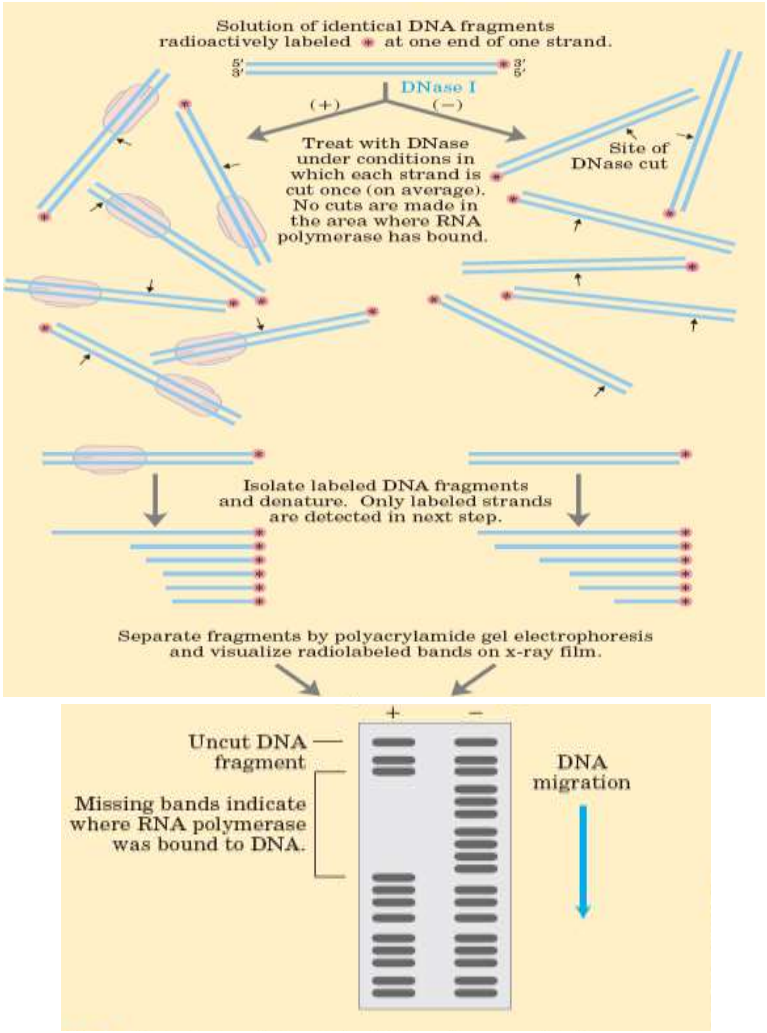
Random amplification of polymorphic DNA (RAPD) is a molecular marker technique using PCR with arbitrary primers for amplifying anonymous stretches of DNA (Chang & Meyerowitz, 1991). Southern blotting is a method of detection of specific DNA sequences in DNA samples. A southern blot combines the transfer of electrophoresis-separated DNA fragments to filter membranes and subsequent fragment detection by probe hybridization (Bignon, Roux-Dosseto, et.al., 1990). With regards to in vitro enzymatic amplification of DNA, the polymerase chain reaction (PCR) has developed into one of the most promising methods allowing widespread applications in DNA cloning, sequencing and mutagenesis related studies (Appa Rao, Mohan, et.al., 1994).

Conclusion:

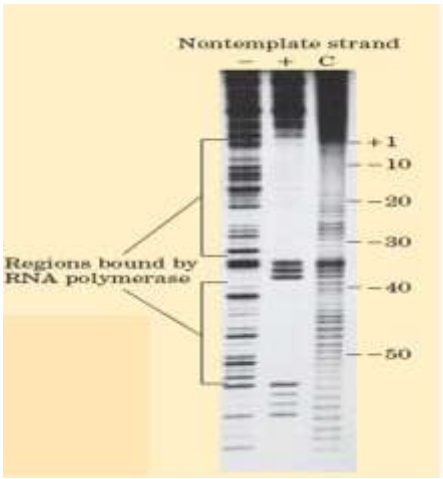
Restriction mapping is a technology to identify the unknown genes without sequencing. It has enabled to industry of biotechnology to its riches now. Though there are many techniques that replaced restriction mapping, it is still in use for low-cost academic exercises and other experiments. It has contributed heavily to our knowledge of DNA manipulation studies. It is an essential tool in comparing DNA fragments together. Many of the technologies like, RFLP, RAPD, PCR, HTGS have replaced restriction mapping nowadays though it would be immature to underestimate the value of restriction mapping.

DNA Footprinting

Footprinting**,** a technique derived from principles used in DNA sequencing, identifies the DNA sequences bound by a particular protein. Researchers isolate a DNA fragment thought to contain sequences recognized by a DNA-binding protein and radiolabel one end of one strand. They then use chemical or enzymatic reagents to introduce random breaks in the DNA fragment (averaging about one per molecule). Separation of the labeled cleavage products (broken fragments of various lengths) by high-resolution electrophoresis produces a ladder of radioactive bands. In a separate tube, the cleavage procedure is repeated on copies of the same DNA fragment in the presence of the DNA-binding protein. The researchers then subject the two sets of cleavage products to electrophoresis and compare them side by side. A gap (“footprint”) in the series of radioactive bands derived from the DNA protein sample, attributable to protection of the DNA by the bound protein, identifies the sequences that the protein binds. The precise location of the protein binding site can be determined by directly sequencing copies of the same DNA fragment and including the sequencing lanes (not shown here) on the same gel with the footprint. Footprinting results for the binding of RNA polymerase to a DNA fragment containing a promoter. The polymerase covers 60 to 80 bp; protection by the bound enzyme includes the -10 and -35 region



Footprint analysis of the RNA polymerase–binding site on a DNA fragment. Separate experiments are carried out in the presence (+) and absence (-) of the polymerase.



Footprinting results of RNA polymerase binding to the lac promoter. In this experiment, the 5’end of the nontemplate strand was radioactively labeled. Lane C is a control in which the labeled DNA fragments were cleaved with a chemical reagent that produces a more uniform banding pattern.

Methyl interference assay:
The methylation interference assay is an analytical method that is used to determine which nucleotides in a DNA molecule are important for protein binding. This method provides information on where protein binding sites are and what proteins bind to a specific DNA molecule. Thus providing insight into how a gene may be regulated.

History and Significance:
The methylation interference assay was developed from the Maxam-Gilbert DNA sequencing method which also uses the methylation of guanine and adenine nucleotides followed by cleavage of these modified residues by piperidine. This technique was originally used to determine which factors were involved in modulating expression of the immunoglobin gene but has been used to study a wide range of problems from disease susceptibility to muscle function. It is especially useful due to its ability to resolve single nucleotides and is one of the highest resolution methods used to investigate sequence specific DNA-protein interaction.

Technique
Initially DNA fragments are ^{32}P end labeled after which guanine and adenine nucleotides are methylated by treatment with dimethyl sulfate. Treatment with this chemical generally results in 1 modified base per DNA molecule. Next the protein of interested is incubated with the methylated DNA fragment. If the DNA is modified at nucleotides that are involved in protein binding, the protein will be unable to bind to the DNA. However, if methylation has occurred at nucleotides that are not important to protein binding, the protein will be able to bind to the DNA. DNA fragments that are bound by protein and those that are not bound by protein can be separated via electrophoretic mobility shift assay. Other common techniques used to separate bound and unbound DNA fragments include filter binding and immunoprecipitation. Next the DNA molecules are treated with an enzyme, such as piperidine, which will cleave the DNA molecules into smaller fragments. Piperidine cleaves DNA at modified bases. Finally the fragments are run on a denaturing gel along with a sequencing ladder. Because each DNA molecule is only methylated at a single position the sequencing ladder can be used to determine which nucleotide had been modified. Cleavage fragments that are generated from unbound DNA will differ from the fragments generated from DNA that was bound by protein. Thus the fragments generated from bound or unbound DNA can be used to determine the nucleotides that are important in protein binding.

Gene Amplification: Polymerase Chain Reaction (PCR):
PCR provides a simple and ingenious method for exponential amplification of specific DNA sequences by in vitro DNA synthesis, i.e., this technique has made it possible to synthesize large quantities of DNA fragments without cloning it.

Kary Mullis in 1985 developed the technique based on the use of an enzyme which is named as Taq DNA polymerase. The PCR technique has now been automated and is carried out by a specially designed machine.

Technique:

The technique involves the following three steps (Fig. 22.16):

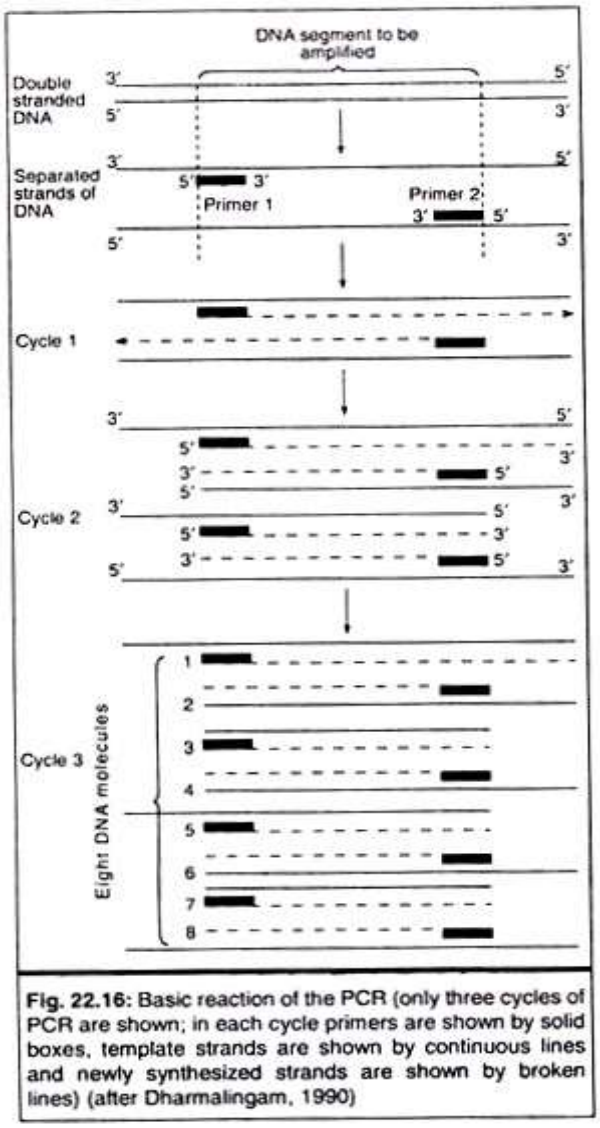


Fig. 22.16: Basic reaction of the PCR (only three cycles of PCR are shown; in each cycle primers are shown by solid boxes, template strands are shown by continuous lines and newly synthesized strands are shown by broken lines) (after Dharmalingam, 1990)

i. Denaturation of DNA Fragment:

The target DNA containing sequence to be amplified is heat denatured (around 94°C for 15 sec) to separate its complementary strands, this process is called melting of target DNA.

ii. Annealing of Primers:

Primers are added in excess and the temperature is lowered to about 68°C for 60 sec., as a result the primers form the hydrogen bonds and anneal to the DNA on both sides of the DNA sequence.

iii. Primer Extension:

Finally different nucleoside triphosphate (dATP, dGTP, dCTP, dTTP) and a thermo-stable DNA polymerase (Taq polymerase from Thermus aquaticus and Vent polymerase from *Thermococcus litoralis*) are added to the reaction mixture, it helps in polymerization process of primers and, therefore, extends the primers (at 68°C) resulting in synthesis of multiple copies of target DNA sequence.

After completion of all these steps in one cycle, again the second cycle is repeated following the same process. If 20 such cycles occur, then about one million copies of target DNA sequence are produced. Recently this technology has been improved much more, where instead of Taq polymerase the rTth polymerase is used which transcribe RNA to DNA, and thereafter amplify the DNA.

Modified Forms of PCR:
The conventional PCR is the symmetrical PCR technique. There are some other modified forms of PCR which are used for various purposes:

a. AP-PCR (Arbitrarily Primed Polymerase Chain Reaction):
It requires only a single primer of relatively much smaller length compared to the primers used in PCR. This technique is used for DNA profiling, in animal and plant biotechnology as well as in forensic medicine.

b. Asymmetrical PCR:
Target sequences of one strand may be amplified in several orders of magnitude more as compared to its complementary strand. This approach is particularly useful for generating single stranded DNA fragment to be used for sequencing of DNA.

c. I-PCR (Inverted Polymerase Chain Reaction):
In this method it allows the amplification of DNA flanking a known DNA sequence, the primers are facing outwards. Using the inverse PCR, the unknown sequences flanking known sequences can be readily amplified.

d. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction):
Although the PCR amplification is generally performed on the DNA template but using this technique the RNA also can be used for amplification. This technique is particularly useful for studying the expression of genes and for monitoring the obscure species of mRNA.

e. Nested PCR:
Nested PCR primers are ones that are internal to the first primer pair. The larger fragments produced by the first round of PCR is used as the template for the second PCR. This technique eliminates any spurious non-specific amplification products.

Advantages of Polymerase Chain Reaction:
PCR is so sensitive that DNA sequences present in an individual cell can be amplified. The isolation and amplification of a specific DNA sequence by PCR is faster and less technically difficult than traditional cloning methods using recombinant DNA techniques.

Application of PCR in Biotechnology: PCR has many fold applications.

1. The amplification of gene fragments as fast alternative of cloning:
(a) Inserts of bacterial plasmids can be amplified with primers.

(b) DNA from known sequence can be obtained by designing primers.

(c) PCR helps in identification of homologous sequences from related organisms.

(d) Using RT-PCR the 3′ end of cDNA can be amplified (RACE: Rapid Amplification of cDNA Ends).

(e) Reverse PCR helps to know the flanking sequences of a known DNA clone.

2. Modification of DNA Fragments:
Site directed mutagenesis using oligonucleotides as PCR primers provides a powerful approach to study structure-function relation.

3. Diagnosis of Pathogenic Microorganism:
DNA from the infected parts of a person or animal may be subjected to PCR with primer specific gene of the pathogen and diagnosis can be done on amplification of DNA.

4. DNA Analysis of Archaeological Specimens:
As DNA is relatively stable and remain intact for a long period of time, PCR can help in analysis of DNA from those embedded materials.

5. Detection of Mutation Relevant for Inherited Diseases:
Any point mutation, a deletion or an insertion and expanded tandem trinucleotide repeat can be detected by PCR. Somatic mutations in oncogenes or tumour repressor genes can also be detected by PCR with primers flanking the insertions or deletions.

6. Analysis of Genetic Markers for Forensic Applications, for paternity testing and for the mapping of hereditary traits.

(a) Amplification of SSR.

(b) RAPD (Random Amplified Polymorphic DNA) with arbitrary, often short (10 bp) primers.

7. Species-Specific Amplification of DNA Segments between interspersed repeat elements (IRS) using the primer based on the SINE sequence (Short Interspersed Nuclear Elements).

8. Genetic Engineering using PCR:
Using PCR we can incorporate alteration or mutation in the ultimate product by choice altering, removing or adding sequences to the primer at the 5′ end. By recombinant PCR technique, it is possible to join two DNA fragments at a specific site through complementary overlaps (This technique is termed as splicing). By synthesizing two mutagenic primers, spanning the internal site to be changed, it is possible to introduce mutations within a fragment.

Probable Questions:

1. Differentiate among Type I, II and III restriction endonuclease.
2. What is star activity? Give example.
3. Define isoschizomer. Give example.
4. How nomenclature of a type II RE is done?
5. Explain blunt cut and staggered cut of RE.
6. Hoe RE are used in genetic engineering ?
7. Explain the role of alkaline phosphatase in RDT.
8. Explain the role of DNA ligase in RDT.
9. Explain the role of Reverse transcriptase in RDT.
10. Explain the role of S1 nuclease in RDT.
11. Explain the role of RNase H in RDT.
12. What is restriction mapping? How it is performed?
13. What is DNA footprinting? What is its significance?
13. What is methyl interference assay. Explain the procedure.
14. Define PCR. Describe different steps of PCR.
15. Discuss application of PCR in biotechnology.
16. Explain nested PCR, asymmetrical PCR, AP-PCR, RT-PCR in brief.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
9. Primrose SB and Twyman RM. (2006) Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
10. Sambrook J and Russell D. (2001) Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Pres

UNIT-V

Basic biology of cloning vectors: plasmids, phages, single stranded DNA vectors, high capacity vectors, retroviral vectors, expression vectors and other advanced vectors in use. Gene cloning strategies: methods of transforming E. coli with rDNA; methods of selection and screening of transformed cells; construction of genomic and cDNA libraries; phage display

Objective: In this Unit we will discuss about different types of cloning vectors and gene cloning strategies also. In this unit you will know about different methods of transformed cell selection and also have a idea about expression of foreign genes.

Cloning Vector:

By cloning, one can produce unlimited amounts of any particular fragment of DNA. In principle, the DNA isolated and cut pieces are introduced into a suitable host cell, usually a bacterium such as Escherichia coli, where it is replicated, as the cell grows and divides. However, replication will only occur if the DNA contains a sequence which is recognized by the cell as an origin of replication. Since such sequences are infrequent, this will rarely be so, and therefore, the DNA to be cloned, has to be attached to a carrier, or vector DNA which does contain an origin of replication.

Criteria of an Ideal Vector:

Vectors are those DNA molecules that can carry a foreign DNA fragment when inserted into it. A vector must possess certain minimum qualifications to be an efficient agent for the transfer, maintenance and amplification of the passenger DNA.

1. The vector should be small and easy to isolate.
2. They must have one or more origins of replication so that they will stably maintain themselves within host cell.
3. Vector should have one or more unique restriction sites into which the recombinant DNA can be inserted.
4. They should have a selectable marker (antibiotic resistance gene) which allows recognition of transformants.
5. Vector DNA can be introduced into a cell.
6. The vector should not be toxic to host cell.

Types of Vector:

Based on the nature and sources, the vectors are grouped into bacterial plasmids, bacteriophages, cosmids and phagemids (Fig. 22.3).

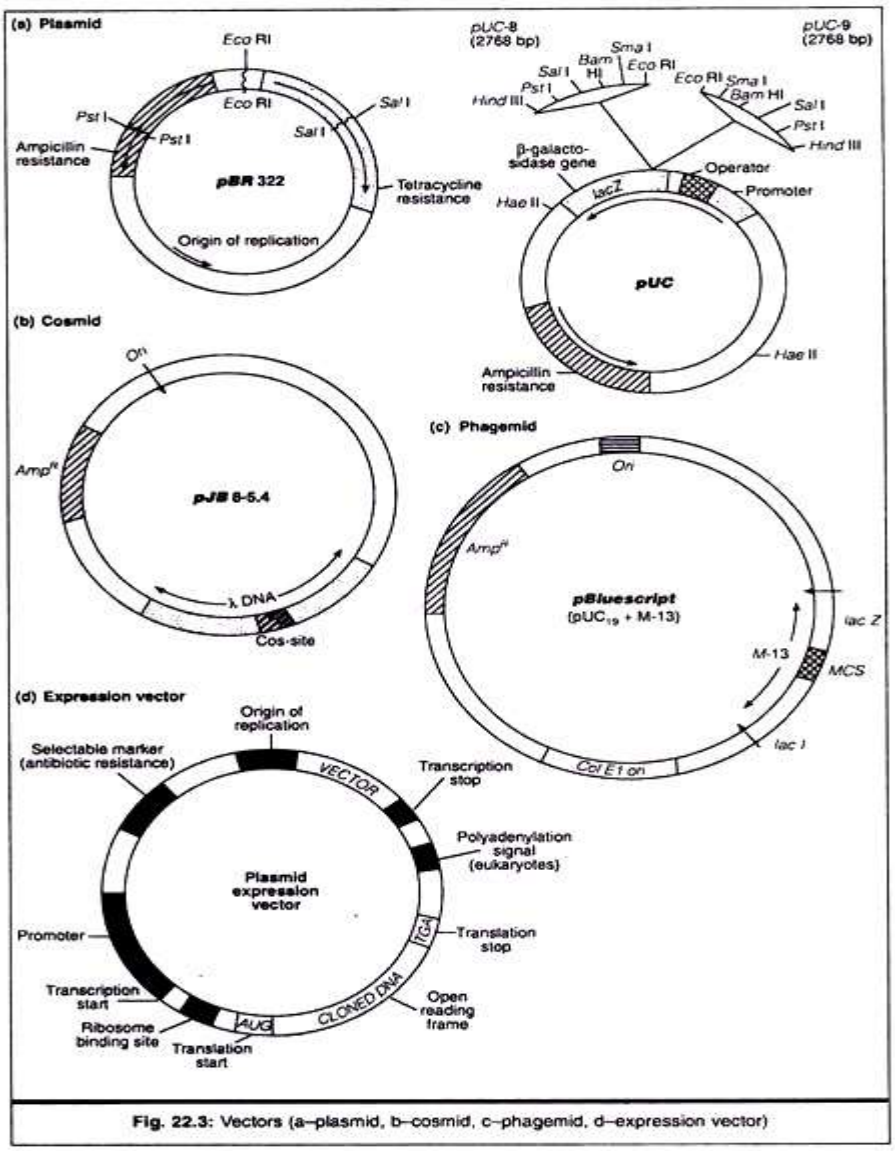


Fig. 22.3: Vectors (a–plasmid, b–cosmid, c–phagemid, d–expression vector)

A. Plasmid:

Plasmids are the extra chromosomal genetic elements commonly found in bacteria and are mostly made of double-stranded circular DNA. They are used as vectors in gene cloning, because they have a replication origin in their DNA making them competent of autonomous replication, and also because they generally have one or two restriction sites for several restriction enzymes.

Many plasmids have been genetically engineered to add useful properties. The number of copies of plasmids may vary from one to several per host cell. Some plasmids under relaxed replication control can have larger number of copies which may be sometimes as high as 1,000. Such high copy-number plasmids are specially suitable for cloning.

A commonly used small cloning vector is the plasmid pBR 322 which has a circular double-stranded DNA having 4,363 base pairs, compared to the E. coli chromosome having about 4,700 x 10^3 base pairs. It carries single restriction sites for the restriction enzymes EcoR1, Hind III, Bam HI and Sal I. Thus, each of these enzymes can make single cleavages at their respective sites of pBR 322 DNA, where the foreign DNA can be inserted.

Many plasmids also have genes for antibiotic resistance which can be profitably used as selectable markers. For example, pBR 322 has two genes specifying resistance to ampicillin

(amp^r) and tetracycline (tet^r). Presence of two such marker genes is more advantageous than a single marker, provided one of the marker genes has a restriction site within it. When a foreign DNA fragment is inserted within the resistance gene, it results in the inactivation of the resistance gene, just as a transposon causes inactivation of a gene.

Such inactivation of an antibiotic resistance gene makes the host ceil in which the plasmid is present susceptible to the particular antibiotic. This property can be utilized for identification and selection of the host cells in which the foreign gene has been cloned. For example, pBR 322 possesses a restriction site for Hind III in the tet^r gene.

When a foreign gene is cloned using Hind III in pBR 322, tet^r gene is inactivated and the host cells containing the recombinant plasmid show resistance to ampicillin because the amp' gene is intact, but not to tetracycline. This makes possible the identification of the host cells containing the cloned foreign gene, because they cannot grow on a medium in which tetracycline has been incorporated at an inhibitory concentration. Other host cells which take up only the vector DNA without the cloned gene will grow in such a medium, because their tet^r gene is intact.

Naturally occurring plasmids do not usually possess all the desirable properties which are useful for their use as cloning vectors. So they must be suitably altered by molecular biological techniques. For example, the plasmid pBR 322 was developed by several alternations. Other plasmids which are used as cloning vectors include pSC 101, pUC 8, pHC 79 etc. (p stands for plasmid).

A very useful plasmid used as a vector for introducing foreign genes into plants is the Ti-plasmid of a plant pathogenic bacterium, Agrobacterium tumefaciens which infects many dicotyledonous plants causing a disease, known as crown-gall. When this organism infects a host plant, it transfers naturally a portion of the Ti-plasmid into the plant. This portion is known T-DNA. The T-DNA segment has been extensively altered by molecular biological methods to make the Ti-plasmid suitable as a vector for inserting foreign DNA into many plants to produce transgenic plants.

Plasmids have following advantages as cloning vehicle (Cohen et a. 1973):

1. It can be readily isolated from the cells.
2. It possesses a single restriction site for one or more restriction enzymes.
3. Insertion of foreign DNA does not alter the replication properties.
4. It can be reintroduced into cell.
5. Selective marker is present.
6. Transformants can be selected easily by using selective medium.
7. Multiple copy numbers are present in a cell.

Some plasmid vectors are pBR 322, pBR 327, pUC vectors, yeast plasmid vector and Ti, Ri plasmids. Ti and Ri Plasmids are widely used in plant system for genetic transformation.

Among higher plants, Ti plasmid of Agrobacterium tumefaciens or Ri plasmid of *A. rhizogenes* are the best known vectors. T-DNA, from Ti or Ri plasmid of Agrobacterium, is considered to be very potential for foreign gene transfer in cloning experiments with higher plants.

pBR 322 and pUC Vectors:

pBR322 is a derived plasmid from a naturally occurring plasmid col El, composed of 4362 bp DNA and its replication may be more faster. The plasmid has a point of origin of replication

(ori), two selectable marker genes conferring resistance to antibiotics, e.g., ampicillin (amp^r), tetracycline (tet^r) and unique recognition sites for 20 restriction endonucleases.
Tetracycline resistance gene has a cloning site and insertion of foreign segment of DNA will inactivate the tet^r gene. The recombinant plasmid will allow the cells to grow only in presence of ampicillin but will not protect them against tetracycline .
Another plasmid used in gene cloning is pUC vector available in pairs with reverse orders of restriction sites relative to lac^z promoter. This is a synthesized plasmid possessing ampicillin resistance gene (amp^r), origin of replication from pBR322(on) and lac^z J gene from E. coli. pUC 8 and pUC 9 make one such pair.

B. Bacteriophage as Cloning Vectors:

The plasmid based vectors used for cloning DNA molecules generally carry up to 10 kb of inserted DNA. However, for the formation of library, it is often helpful to be able to maintain larger pieces of DNA. For this reason, E. coli virus (Bacteriophage, phage) lambda (λ) has been developed as a cloning vehicle. In its life cycle, bacteriophage λ infects E. coli and after injection of the viral DNA, two possibilities exist.
Bacteriophage λ can enter a lytic cycle, which after 20 minutes lead to the lysis of host cells and the release of about 100 phage particles. Alternatively, the injected bacteriophage λ DNA can be integrated into the E. coli chromosome (DNA) as a prophage and can be maintained more or less indefinitely (Lysogeny stage).
However, under conditions of nutritional or environmental stress, the integrated bacteriophage λ DNA can be excised and enter a lytic cycle. The bacteriophage λ DNA is about 50 kb in length, of which approximately 20 kb is essential for the integration excision (I/E) events. For forming genomic libraries, 20 kb of DNA can be replaced with 20kb of cloned DNA.

Cloning Vectors based on the Bacteriophage Lambda (λ):
Derivatives of the genome of bacterioplage lambda have been constructed to serve as cloning vectors. Transfection or transduction is used to introduce such vectors into E. coli.

Two properties of the lambda genome make it suitable for use as a vector:
1. Only about 50% of the 50 genes of lambda are essential for its replication and for lysis of the host cell. Most of these non essential genes are located together in a cluster around the middle of the genome.
2. Lambda genome is packaged inside the phage head by what is known as the 'head-full mechanism'. This means that not only there is an upper limit of the amount of DNA that is packaged inside the phage head, but there is a lower limit also. Effective packaging takes place only when a minimum amount of DNA is present, i.e., 35 kb (kilo = thousand base).
An infective bacteriophage λ consists of a tubular protein tail with a few tail fibres and a protein head. The production and assembly of heads and tails, and packaging of DNA are highly coordinated sequence of events. The DNA within head of a λ phage is a 50 kb linear molecule with a 12-base, single stranded extension at the 5′ end. These extensions are called cohesive (cos) ends, because they contain sequences that are complementary to each other.

In E. coli, these cos ends base pair to form a circular DNA. DNA replication from the circular DNA creates a linear form of λ DNA that is composed of several contiguous lengths of 50 kb units. Each new assembly is filled with 50 kb DNA (Fig. 14.3).

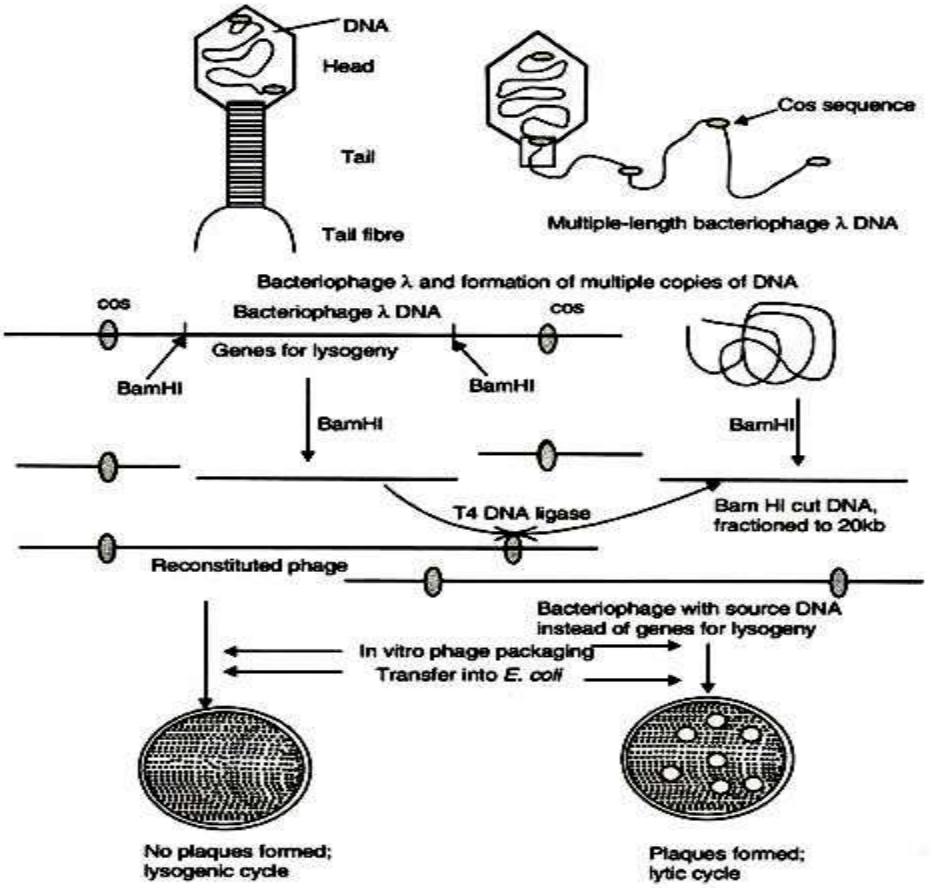


Fig. 14.3. Bacteriophage cloning system.

a. Inserting Type Lambda (λ) Vectors:

A lambda cloning vector can therefore be constructed by deletion (in vivo or in vitro by restriction deletion) of a part of the non-essential region such that the remainder is not less than 35kb. Other mutations are also introduced such that restriction sites in the enemies regions are eliminated. A segment of foreign DNA can be cloned in an unique restriction site in the non-essential region, the only condition being that the vector and the insert together would not to be more than 53 kb long. Such vectors are termed "insertion vectors".

Some of the Charon vectors are examples of this type of vectors. Bacteriophage X cloning vector has two Bam HI sites that flank the I/E region. When this DNA is cut with Bam HI, three segments are produced. The middle segment I/E region, which is replaced by cloned DNA of 20 kb size. The source is cut with Bam HI, and DNA pieces that are 15 to 20 kb in length are isolated. The two DNA samples (phage and source) are combined and incubated with T4 DNA ligase.

Then empty bacteriophage heads and tail parts are added. Under these conditions 50 kb unit of DNA are packed in to the heads, and infective phages are produced. Other products from ligation reaction cannot be packed, because they are either too large (>52 kb) or too small (>38 kb). Recombinant bacteriophage λ can undergo lytic cycle only in an E. coli strain that does not allow reconstituted phage λ (non-recombinant) with intact I/E regions to grow.

Recombinant phage is maintained by lytic cycles in fresh E. coli cultures. Bacteriophage libraries can be screened by using either DNA probes or immunological assays. For this purpose, individual lytic zones are tested (compared to bacterial colonies in plasmid cloning vectors).

b. Substitution Type Lambda (λ) Vector:

The second type of lambda vector is of the substitution type, the example being the lambda gt vectors and the EMBL vectors. These vectors have two Eco R1 sites or two BamHI sites in the non-essential region.

On digestion with EcoRl, at least three piece(s) are produced, two terminal ones containing the essential regions and the central piece(s) containing the non-essential genes.

The central piece (s) is separated out by sucrose density gradient sedimentation and replacing by the foreign segment to be cloned. The limits of the size of foreign DNA that can be cloned in the lambda gt vector is 1-14 kb and in the Charon 4 vector is 8.2-22.2 kb.

Such a replacement cloning vector has an advantage over the insertion vectors. The terminal pieces by themselves if joined by DNA ligase, do not make up 35 kb and hence cannot be packaged. Packaging occurs only when a segment of foreign DNA gets cloned between the two terminal pieces of the vector and hence no separate selection for recombination molecules to is necessary.

C. Phagemids as Cloning Vectors:

A phagemids is a hybrid of a plasmid and a filamentous coliphage that can be propagated in either form. The coliphage could be either of the three virtually identical phages, M13 fd or f1. These are male specific phages that contain single stranded circular DNA as their genome. Upon infection of E. coli by the bacteriophage, double stranded DNA is first formed as the replicative intermediate.

Finally single stranded DNA is packaged into the virion. Both the replication origins of the plasmid and the coliphage are incorporated in the phagemid. The auxiliary replication functions necessary in trans for the coliphage replication are not, however, incorporate in the phagemid. Hence, replication from the coliphage origin can take place only in the presence of a helper phage.

Otherwise, replication takes place from the plasmid replication origin. The pBLUESCRIPT phagemids have both Col E, (pMB 9 like) origin and the filamentous phage f1 origin. The cloning is done in any of the multiple cloning sites in the double stranded circular DNA of the plasmid form of the vector. This is introduced in to E.coli by transformation and the synthesis of single stranded DNA from the phage fl origin is induced by superinfection with a helper phage. The single stranded DNA formed is packaged in to the phage rods because of the presence of the phage packaging signals as well in the phagemid. Direct base sequencing can be undertaken using the single stranded DNA isolated from the virions secreted from the E. coli cells. Depending on the orientation of the f1 replication origin in the phagemid, either the (+) strand or the (-) strand of the phagemid is replicated in presence of the helper phage. The pEMBL phagemids are similar to the pBLUESCRIPT phagemids.

Phagemids that have replication origin of pUC plasmids and of the M13 bacteriophage have also been developed and are available under the trade name of LITMUS vectors. Replication in the circular double stranded plasmid form uses the pUC replication origin. Cloning is done in the multiple cloning sites located in the lacZ gene and the usual blue/white selection is

available. Here also replication of the single stranded phage DNA form is induced by super infecting with the M13 helper phage.

D. Cosmids as Cloning Vectors:

Plasmid vectors are not suitable for cloning DNA fragments very much larger than their own size, as the transformation frequency fall beyond acceptable limits and cloned fragments or their parts very often get deleted. Takagi and co-workers observed as early as 1976 that the presence of the cohesive end site cos λ from the bacteriophage lambda DNA in a plasmid allows it to be packaged in vivo into virus particles. The interesting finding was that the in vivo packaging mechanism would be select DNA molecules of the full size of the lambda genome (~48.5 kb). Making use of this finding, cosmid vectors were first developed in 1978 by J. Collins and coworkers to facilitate cloning of larger DNA fragments in plasmids. Extracts of lambda lysogens have been successfully used for in vitro packaging of the lambda capsids. An example of a commonly used cosmid is pHV79 which is nothing but pBR322 containing the cohesive end site cos λ and which can accommodate up to 45 kb sized inserts.

A great advantage of such a cosmid vector is that:

(1) Gene libraries consisting of a smaller number of clone members can span the whole genome of an organism. For example, the genome of Escherichia coli can be accommodated in just 120 cosmids.

(2) Other advantages are that large gene can be studied intact and genetic linkage studies can be carried out at the molecular level.

(3) An important practical advantage of a cosmid is that background molecules which do not have the intact and genetics linkage studies can be carried out at the molecular level.

(4) An important practical advantage of a cosmid is that background molecules which do not have inserts or have smaller inserts are eliminated during packaging. This is not possible to achieve with plasmid cloning vectors.

(5) Besides, the frequency of transformation of the lambda capsids with an in vitro packaging extract is much higher than the transformation frequency of plasmids.

Cosmid cloning vectors can carry 40 kb of cloned DNA and can be maintained as plasmids in E. coli. Cosmids combine the properties of plasmids and bacteriophage λ vectors. The commonly used cosmid pLFR-5 (6kb size) has two cos sites (cos ends) from bacteriophage λ separated by a Sea I restriction endonuclease site, a multiple cloning sequence with six unique sites (Hind III, PstI, Sail, BamHI, SmaI, and EcoRI), an origin of DNA replication (ori) and a tetracycline resistance (Jet) gene. This cosmid carry about 40 kb of cloned DNA.

For this vector, pieces of DNA that are approximately 40 kb in length are purified by sucrose density gradient configuration from a partial digestion of source DNA with Bam HI. The pFLR-5 DNA is cleaved first with Seal and then with Bam HI. The two DNA samples are mixed and ligated. Some of the ligand products will have a 40kb DNA piece inserted between the two fragments that are derived from the digestion of the pLFR-5 DNA.

The molecules formed by joining will be about 50 kb long, with cos sequences that are about 50 kb apart. Therefore, these DNA constructs can be successfully packaged into bacteriophage λ heads in vitro (as described above, phage λ head accommodate only 50kb DNA). After formation of complete phage, the DNA is delivered by infection into E. coli.

During phage packaging, cos ends are cleaved. Once inside the bacteria, the cos ends base pair and form a circular DNA molecules (Fig. 14.4). This circular form is stable, so the cloned DNA can be maintained as a plasmid-insert DNA construct because the vector contains a complete set of plasmid functions. Moreover, the tetracycline resistance gene allows colonies that carry the cosmid to grow in presence of tetracycline; non-transformed cells are sensitive to tetracycline and die.

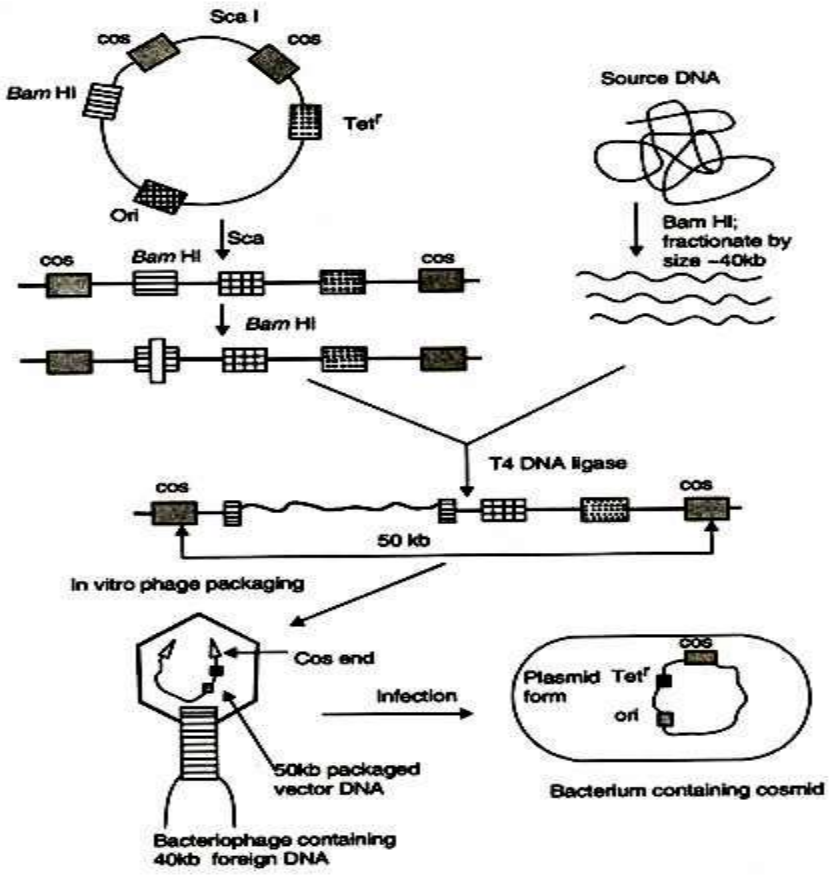


Fig. 14.4. Cosmid vector.

The following are the steps for construction of a cosmid library:

(i) Cleavage of the genome by partial digestion with restriction endonuclease,

(ii) Sizing of the fragments by gel electrophoresis or velocity centrifugation;

(iii) Cleavage of the cosmid vector and treatment with phosphate to minimize polycosmid formation;

(iv) Ligation of the genomic DNA and the cosmid DNA;

(v) Packaging the ligated DNA into infectious phage particles;

(vi) Transduction into E. coli.

Therefore, it is evident that different vectors have different capacity of carry foreign DNA

E. Bacteriophage M13 vectors:

General Biology:

The M13 family of vectors is derived from bacteriophage M13. This is a male specific (infects E. coli having f. pili), lysogenic filamentous phage with a circular single-stranded DNA genome about 6,407 bp (6.4 kb) in length. Once inside the host-cell the single-stranded DNA

of M13 phage acts as the template for synthesis of a complementary strand, resulting in normal double-stranded DNA [Fig. 4.23(a)].

This molecule is not inserted into the bacterial genome, but instead replicates until over 100 copies are present in the cell [Fig. 4.23(b)]. When the bacterium divides, each daughter cell receives copies of the phage genome, which continues to replicate, thereby maintaining its overall numbers per cell.

As shown in [Fig 4.23(c)], new phage particles are continuously assembled and released, about 1000 new phages being produced during each generation of an infected cell.

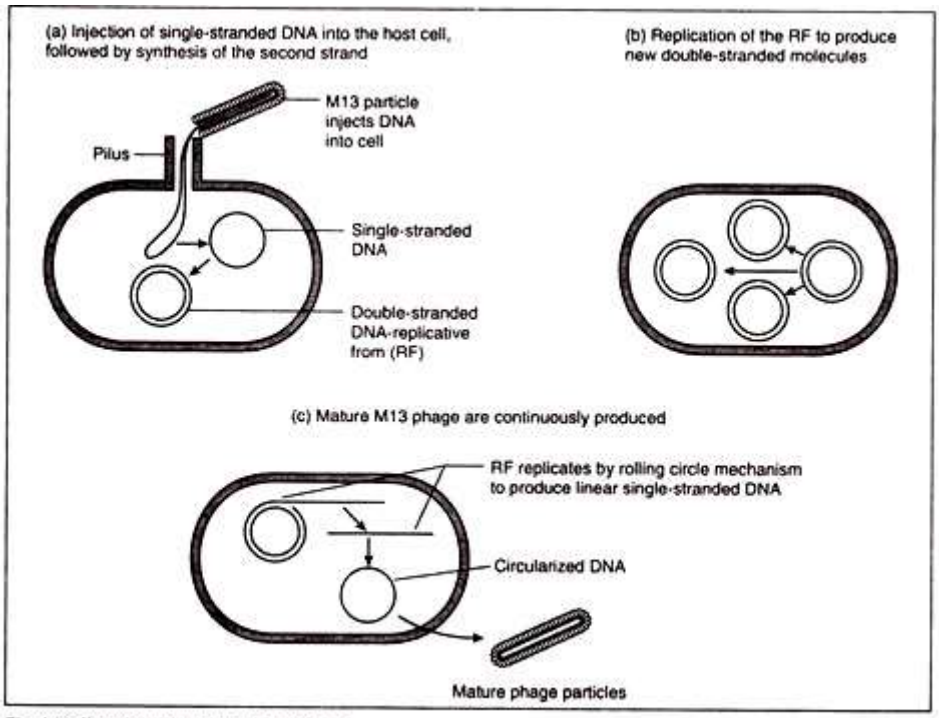


Fig. 4.23: The life cycle of M13 bacteriophage

The Attraction of M13 as a Cloning Vector:

Several features of M13 make this phage attractive as the basis for a cloning vector. The genome is less than 10 kb in size, well within the range desirable for a potential vector. In addition, the double-stranded replicative form (RF) of the M13 genome behaves very much like a plasmid, and can be treated as such for experimental purposes.

It is easily prepared from a culture of infected E. coli cells and can be reintroduced by transfection. Most importantly, genes cloned with an M13-based vector can be obtained in the form of single- stranded DNA. Single-stranded version of cloned genes are useful for several techniques, notably DNA sequencing and in vitro mutagenesis.

Using an M13 vector is an easy and reliable way of obtaining single-stranded DNA for this type of work.

Construction of M13 vectors:

The first step in the construction of M13 cloning vector is to introduce the lac Z' gene into the inter-genic sequence. This gives rise to M13 mp1 which forms blue plaques on X-gal agar (Fig 4.25(a)) M13 mp1 does not progress any unit restriction site in the lac Z' gene.

It however, contains a hexanucleotide sequence GGATTC near the start of the gene. A single nucleotide change (by using in vitro mutagenesis) would make this GAATTC, which is an EcoR1 site.

This results in the formation of M13 mp2. M13 mp2 has a slightly altered lac Z' gene but the beta-galactosidase enzyme produced by cells infected with M13 mp2 is still perfectly functional. M13 mp2 is the simplest M13 vector. DNA fragments with ECoR1 sticky ends can be inserted into the cloning site and recombinants are distinguished as clear plaques on X-gal agar.

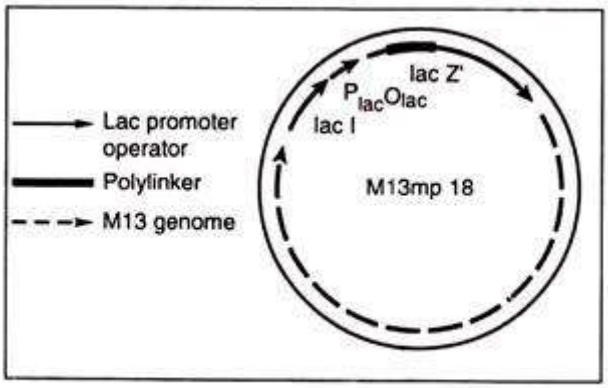


Fig. 4.24: M13 vector

We go for further modifications of M13 mp2 resulting in the production of another M13 vector called M13 mp7. In the generation of M13 mp7 first of all we synthesize a short oligonucleotide called poly-linker that consists of a series of restriction sites and has EcoR1 sticky ends. This poly-linker is inserted into the EcoRI site of M13 mp2 to generate M13mp7.

This poly-linker also provides as many as four possible cloning sites (ECoRI, BamHl, SaiI and PstI) to the new vector. It is very important to note that the poly-linker is designed so that it does not totally disrupt the lac Z' gene: a reading frame is maintained throughout the poly-linker, and a functional, though altered, beta-galactosidase enzyme is still produced.

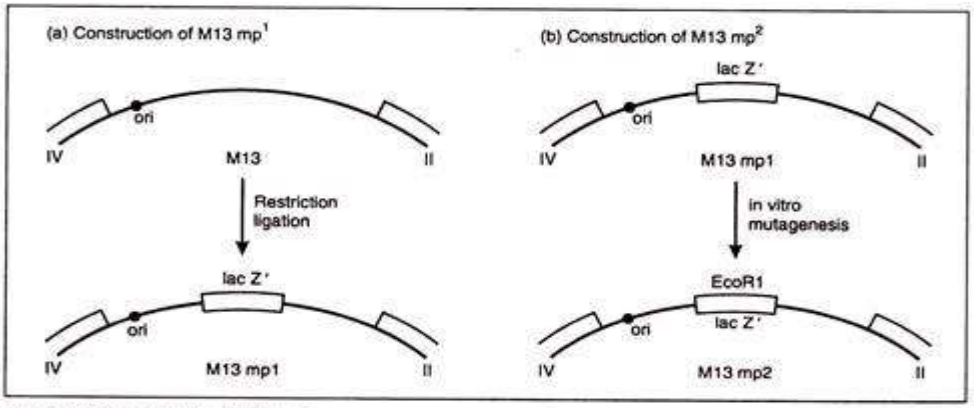


Fig. 4.25: The construction of M13mp2

Screening of transformed host cells using bacteriophage M13 vectors:
Insertion of new DNA almost invariably prevents beta-galactosidase production. So recombinant plaques are clear on X-gal agar. Alternatively, if the poly-linker is reinserted, and the original M13mp7 reformed, then blue plaques result.

Uses of bacteriophage M13 Vectors:

1. DNA Sequencing:
For a long time the most important application of M13 cloning was in DNA sequence determination by the Sanger method, also called the dideoxy or chain-termination method. This relies on synthesis of DNA in the presence of chain terminating inhibitors, the 2′, 3′- di-deoxynucleoside triphosphates (ddNTPs). The method is now a very standard tool of molecular biology.

2. Phage Display Vectors:
An important use of filamentous phage is in phage display systems. Here, coding sequences are inserted into one of the coat protein genes. The result is that the phage are generated with a hybrid form of this protein, which is a fusion of the normal protein sequence and the protein product of the inserted sequence (assuming the inserted sequence has the same reading frame as the coat protein gene).
The phages are secreted from the cell, with this extra material 'displayed' on the outside. These display vectors have many uses, e.g., in screening libraries by panning and for vaccine production.

3. Other Applications:
Some protocols for site-directed mutagenesis also use single- stranded DNA, which can be obtained with vectors based on filamentous phages. Single-stranded DNA is also of particular use in generating probes for RNA analysis.

Probes can be prepared that are specific for RNA transcripts from either strand of DNA. The latter applications are outside the scope of this book, but more information can be obtained from specialized laboratory manuals.

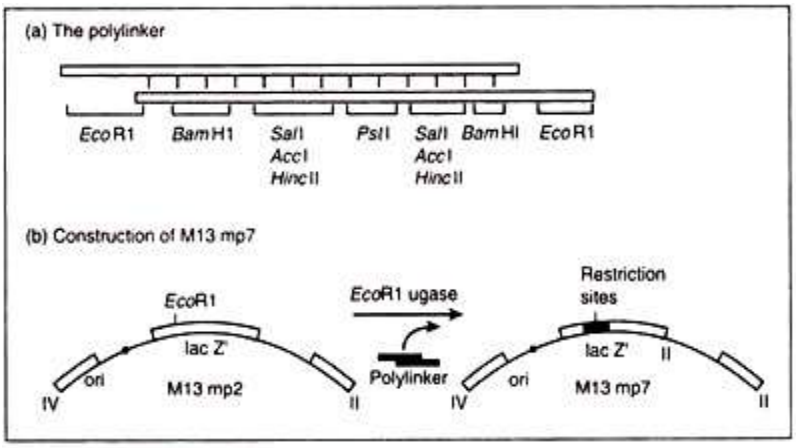


Fig. 4.26: The construction of M13mp7 from M13mp2

Advantages of bacteriophage M13 vector:

1. Advantages over Lambda Phage Vector:

M13 is an example of a filamentous phage and is completely different in structure from lambda. Furthermore, the M13 DNA molecule is much smaller than the lambda genome, being only 6407 nucleotides in length. It is circular and is unusual in that it consists entirely of single-stranded DNA.

The smaller size of the M13 DNA molecule means that it has room for fewer genes than the lambda genome. This is possible because the M13 capsid is constructed from multiple copies of just three proteins (requiring only three genes), whereas synthesis of the lambda head- and-tail structure involves over 15 different proteins.

In addition, M13 follows a simpler infection cycle than lambda , and does not need genes for insertion into the host genome. Injection of an M13 DNA molecule into an E. coli cell occurs via the pilus, the structure that connects two cells during sexual conjugation.

2. Other Advantages:

M13-based vectors are that they contain the same poly-linker and alpha-peptide fragments as the pUC series and recombinants can be selected by the blue → white colour test. Also the size of the genome is below 10 kb and so is easy to handle.

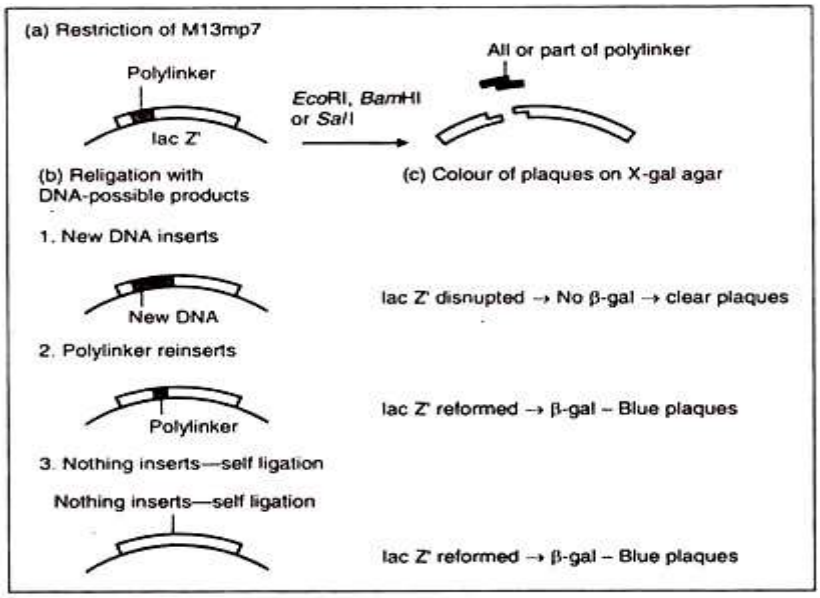


Fig. 4.27: Cloning with M13mp7

Disadvantages of bacteriophage M13 vectors:

The following are the disadvantages of bacteriophage M13 vectors:

1. Gene of interest more than 2kb cannot be cloned.
2. It has low yield of DNA.
3. The phage produce many toxins in high concentration

High Capacity Vectors:

1. Yeast Artificial Chromosomes (YAC):

Cloning of DNA fragments much larger than 45 kb became possible in 1987, when D.T. Burke and G.F. Carle developed in the laboratory of M.V. Olson an altogether new type of yeast vector, which they called yeast artificial chromosome (YAC). The development of YAC's were based on the logic that an eukaryotic linear chromosomes needs for its replication and stability, not only replication origins, but also the centromere and the telomere.

The centromere sequence would attach to the mitotic spindle during cell division and help in efficient segregation of the chromosomes into the daughter cells. The telomere would preserve the integrity of the ends of the linear chromosomes. Once these elements were provided, the vector could be replicate stably like a chromosome and could accommodate chromosomes sized inserts. Indeed, standard YACs can accommodate around 600 kb DNA inserts, while special type of YACs can accommodate up to 1400 kb DNA inserts. Though an YAC vector is meant to be propagated like a chromosome in yeast, it is a circular double stranded DNA that contains a replication origin (colE 1) compatible with E. coli in addition to yeast replication origin or an yeast ARS element. The col El replication origin is useful to a yeast replication origin or an yeast ARS elements. The colEl replication origin is useful for amplification of the vector in E. coli. Next to the yeast replication origin is located the centomere of yeast chromosome 4 (Cen 4). The two telomere sequences are from the protozoan Tetrahymenal, which have been found to be functional in yeast. A staffer element containing the His 3 gene of yeast is present between the two telomere sequences through two BamHI restriction endonuclease sites. Three selectable yeast marker genes, Trp 1, Ura 3 and Sup 4 are also present.

The marker genes Trpl and Ura 3 are on the two sides of the unique restriction endonuclease site SnBl, that produces blunt ends. The SnaB 1 site is used as the cloning site and is located within the Sup4 gene. Sup 4 gene product is a tRNA that suppresses a mutation in the Ade gene of yeast resulting in a change in colour of colonies from red to white in the presence of limiting amounts of adenine in the medium. The YAC vector is digested with BamHI and SnaB I throwing the staffer element out, disrupting the Sup4 gene and yielding two vector fragments termed as left arm and right arm. Each of the arms but at its one end a telomere sequence followed by a BamHI overhang, but a blunt flush cut at the other end. The left arm also has the Cen 4 sequence, the ARS1, the ColEl origin, the amp and Trp 1 markers. The right arm contains the Ura 3 marker. These two arms are blunt end ligated with the long chromosomal DNA fragment from any source, thus creating the artificial linear chromosome among other ligation products. Ligation products are introduced into yeast cells that have mutation in the Trpl, Ura3 and Ade loci by lipofection or by fusion with yeast speroplasts. The yeast cells are allowed to regenerate the cell walls and plated on medium lacking tryptophan and uracil and containing limiting amount of adenine. Only the yeast cells transformed with artificial chromosomes comprising both the left arm and the right arm would grow. The recombinant artificial chromosomes containing the insert would develop into white colonies. The red colonies would represent cells having the linear vector, but no insert. Cells having other ligation products like two left arms or two right arms would not grow.

2. Bacterial Artificial Chromosomes (BAC):

Bacterial artificial chromosomes (BAC) were developed by Mel Simmons and coworkers in the early 1990s and are based on the fertility factor (F factor) of Escherichia coli. The F plasmid, a ~ 100 kb circular double stranded DNA, is present is an E. coli cell in only 1-2 copies. The synthetic BAC vectors, which are only ~7.5 kb double stranded DNA circles contain the replication origin oriS and the gene repE of the F plasmid that are responsible for initiation and proper orientation of replication of the BAC vector. The parA and parB genes of the F plasmid ensure efficient segregation of the F factor into the daughter E. coli cells after its replication are also incorporated in the BAC vector. The BAC vectors also contain multiple cloning sites (mcs), a selectable marker in the form of antibiotic resistance and colour based identification (lac Z complementation system) of recombinants carrying inserts.

The naturally occurring F2 factors consist of up to 25% of the E. coli genome integrated into the basic F factor and are very stable. This characteristic of the F factor contributes to the ability BACs to accommodate very large amount of external DNA to the extent of 300kb. The recombinant BACs have been found to exhibit a lower level of rearrangement and chimerism of the cloned DNA sequence than exhibited by YACs. The cloning of DNA in BACs is done as is done in a plasmid, by linearizing the vector with a restriction endonuclease, treating with phosphatase and then ligating with the DNA fragments to be cloned. E. coli has to be transformed by electroporation because of the large size of the recombinant BAC.

3. Mammalian Artificial Chromosomes (MACs):

The YACs, the BAC and the PACs (plasmid artificial chromosomes) have found regular use for cloning large genomic DNA fragments in various genome sequencing projects. Another potential application of artificial chromosomes is in gene therapy of human. For gene therapy, we need to have gene in human DNA fragments including their promoters and all the control elements. This would have to be introduced into the target cells efficiently and would have to be stably maintained inside the nucleus, generation after generation through unlimited number of divisions. The DNA would have to be expressed properly without interfering with the function of other resident. Such large artificial human chromosome (aptly termed mini-chromosomes), if 1-10 mb size have been claimed to be stable for more than 100 cell generations. Satellite DNA based mini-chromosomes of 20-30 mb have also been reported.

4. Artificial Human Chromosomes:

Researchers at the School of Medicine and Athersys, Inc. have created the first artificial human chromosome. The synthetic chromosomes represent a breakthrough in medical research and provide scientists with a powerful new tool for the study of human genetics. Artificial chromosomes may also offer a new approach to gene therapy and the treatment of a broad range of genetic diseases. A report of the research was published in the April 2007 issue of Nature Genetics. This opens the door to a whole new avenue of research in chromosome biology and gene therapy," said Huntington F. Willard, chairman of genetics at the School of Medicine and University Hospitals of Cleveland. "While it's been known since the early years of this century that chromosomes carry genes, until now the complexity and size of normal chromosomes has limited our ability to analyse their structure and function. The synthetic micro-chromosome system now allows us to perform detailed studies on the nature of chromosomes – essentially the next phase of the Human Genome Project which is to move from just mapping genes to actually understanding how they work and influence human

disease." Inside the cells, the independent elements assembled to form miniature chromosomes, or synthetic micro-chromosomes, that were structurally similar to human chromosomes, but contained less genetic material. Analysis of the newly introduced artificial chromosomes demonstrated normal centromeric activity, genetic stability, and continued gene expression through repeated rounds of the cell cycle.

Expression Vectors or Expression construct:
We use an expression vector when our aim is to obtain the protein product of our gene of interest. To get the protein we need to allow the expression of our gene of interest (hence the name expression vector) by employing the processes of transcription and translation. Apart from the three DNA sequences discussed above (origin of replication, selectable markers and multiple cloning sites), the expression vectors have some special additional sequences as well.

Those are as follows:
a. A bacterial promoter, such as the lac promoter. The promoter precedes a restriction site where foreign DNA is to be inserted, allowing transcription of foreign sequence to be regulated by adding substances that induce the promoter.
b. A DNA sequence that, when transcribed into RNA, produces a prokaryotic ribosome binding site.
c. Prokaryotic transcription initiation and termination sequences.
d. Sequences that control transcription initiation, such as regulator genes and operators.

In some types of expression vectors which are specifically used in association with the bacterial host (like E. coli), multiple cloning site is not immediately adjacent to the ribosome binding sequence, but instead is preceded by a special sequence coding for a bacterial polypeptide.
While using such type of expression vectors the gene of interest is inserted just after the gene for bacterial polypeptide. In this way we fuse two reading frames, producing a hybrid gene that starts with the bacterial gene and progresses without a break into the codons of our gene of interest. The product of gene expression is therefore a hybrid protein, consisting of short bacterial polypeptide fused into amino terminus of our target polypeptide sequence. This hybrid polypeptide chain consisting of two different types of polypeptides is called a fusion protein.

Methods of Gene Transfer in *E. Coli*

Method - 1 Transformation:
Transformation is the method of introducing foreign DNA into bacterial cells (e.g. E.coli). The uptake of plasmid DNA by E.coli is carried out in ice-cold $CaCl_2$ (0-5°C), and a subsequent heat shock (37-45°C for about 90 sec). By this technique, the transformation frequency, which refers to the fraction of cell population that can be transferred, is reasonably good e.g. approximately one cell for 1000 (10^{-3}) cells.

Transformation efficiency:
It refers to the number of trans-formants per microgram of added DNA. For E.coli, transformation by plasmid, the transformation efficiency is about 10^7 to 10^8 cells per microgram of intact plasmid DNA. The bacterial cells that can take up DNA are considered as competent. The competence can be enhanced by altering growth conditions.

The mechanism of the transformation process is not fully understood. It is believed that the $CaCI_2$ affects the cell wall, breaks at localized regions, and is also responsible for binding of DNA to cell surface. A brief heat shock (i.e. the sudden increase in temperature from 5°C to 40°C) stimulates DNA uptake. In general, large-sized DNAs are less efficient in transforming.

Other chemical methods for transformation:

Calcium phosphate (in place of $CaCI_2$) is preferred for the transfer of DNA into cultured cells. Sometimes, calcium phosphate may result in precipitate and toxicity to the cells. Some workers use diethyl amino ethyl dextran (DEAE -dextran) for DNA transfer.

Method - 2 Conjugation:

Conjugation is a natural microbial recombination process. During conjugation, two live bacteria (a donor and a recipient) come together, join by cytoplasmic bridges and transfer single-stranded DNA (from donor to recipient). Inside the recipient cell, the new DNA may integrate with the chromosome (rather rare) or may remain free (as is the case with plasmids). Conjugation can occur among the cells from different genera of bacteria (e.g Salmonella and Shigella cells). This is in contrast to transformation which takes place among the cells of a bacterial genus. Thus by conjugation, transfer of genes from two different and unrelated bacteria is possible.

The natural phenomenon of conjugation is exploited for gene transfer. This is achieved by transferring plasmid-insert DNA from one cell to another. In general, the plasmids lack conjugative functions and therefore, they are not as such capable of transferring DNA to the recipient cells. However, some plasmids with conjugative properties can be prepared and used.

Method – 3 Electroporation:

Electroporation is based on the principle that high voltage electric pulses can induce cell plasma membranes to fuse. Thus, electroporation is a technique involving electric field-mediated membrane permeabilization. Electric shocks can also induce cellular uptake of exogenous DNA (believed to be via the pores formed by electric pulses) from the suspending solution.

Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).

The basic technique of electroporation for transferring genes into mammalian cells is depicted in Fig. 6.11. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.

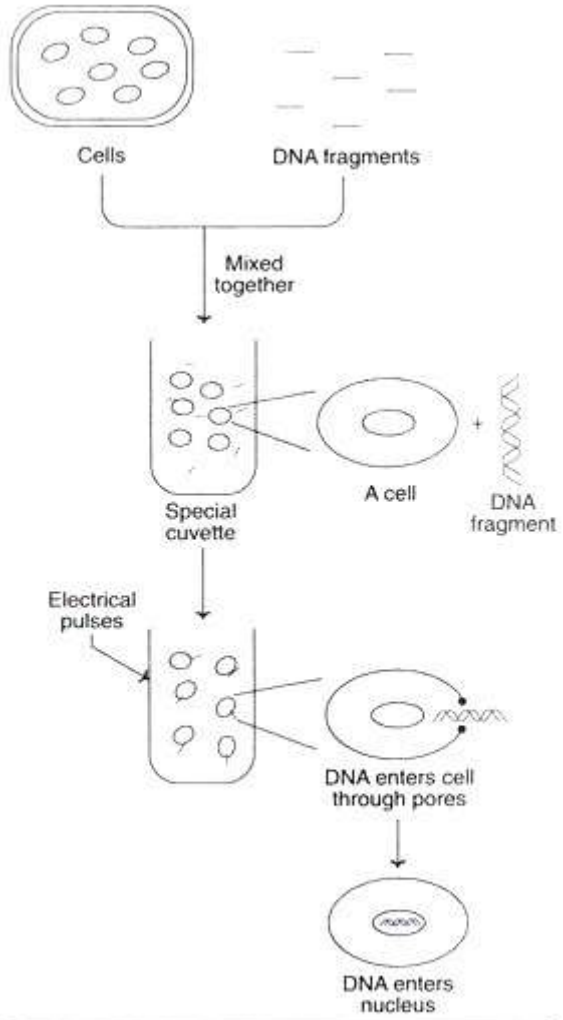


Fig. 6.11 : Gene transfer by electroporation.
(Note : Magnification depicted on right side)

Electroporation is an effective way to transform E.coli cells containing plasmids with insert DNAs longer than 100 kb. The transformation efficiency is around 10^9 transformants per microgram of DNA for small plasmids (about 3kb) and about 10^6 for large plasmids (about 130 kb).

Method # 4. Liposome-Mediated Gene Transfer:

Liposomes are circular lipid molecules, which have an aqueous interior that can carry nucleic acids. Several techniques have been developed to encapsulate DNA in liposomes. The liposome- mediated gene transfer, referred to as lipofection, is depicted in Fig. 6.12.

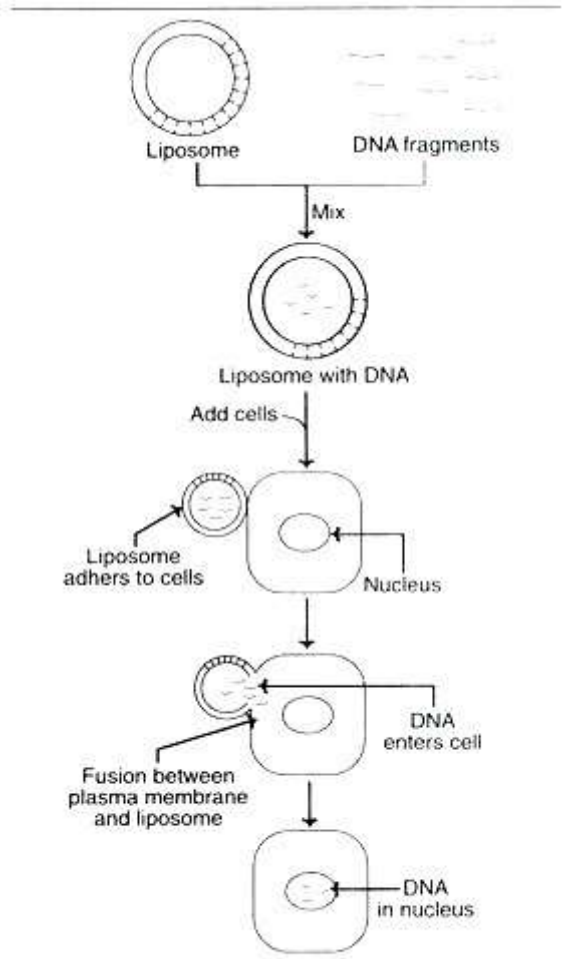


Fig. 6.12 : Liposome-mediated gene transfer
(Note : For clarity, the native cell DNA is not shown).

On treatment of DNA fragment with liposomes, the DNA pieces get encapsulated inside liposomes. These liposomes can adhere to cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. The positively charged liposomes very efficiently complex with DNA, bind to cells and transfer DNA rapidly. Lipofection is a very efficient technique and is used for the transfer of genes to bacterial, animal and plant cells. T

Method # 5. Transduction:
Sometimes, the foreign DNA can be packed inside animal viruses. These viruses can naturally infect the cells and introduce the DNA into host cells. The transfer of DNA by this approach is referred to as transduction.

Method # 6. Direct Transfer of DNA:
It is possible to directly transfer the DNA into the cell nucleus. Microinjection and particle bombardment are the two techniques commonly used for this purpose.

Microinjection:
DNA transfer by microinjection is generally used for the cultured cells. This technique is also useful to introduce DNA into large cells such as oocytes, eggs and the cells of early embryos.

The term transfection is used for the transfer DNA into eukaryotic cells, by various physical or chemical means.

Methods of selection and screening of transformed cells:

The top five screening strategies are: (1) Screening by DNA Hybridization (2) Screening by Colony Hybridization (3) Screening by PCR (4) Screening by Immunological Assay and (5) Screening by Protein Function.

1. Screening by DNA Hybridization:

The target sequence in a DNA can be determined with a DNA probe (Fig. 9.5). To start with, the double-stranded DNA of interest is converted into single strands by heat or alkali (denaturation). The two DNA strands are kept apart by binding to solid matrix such as nitrocellulose or nylon membrane.

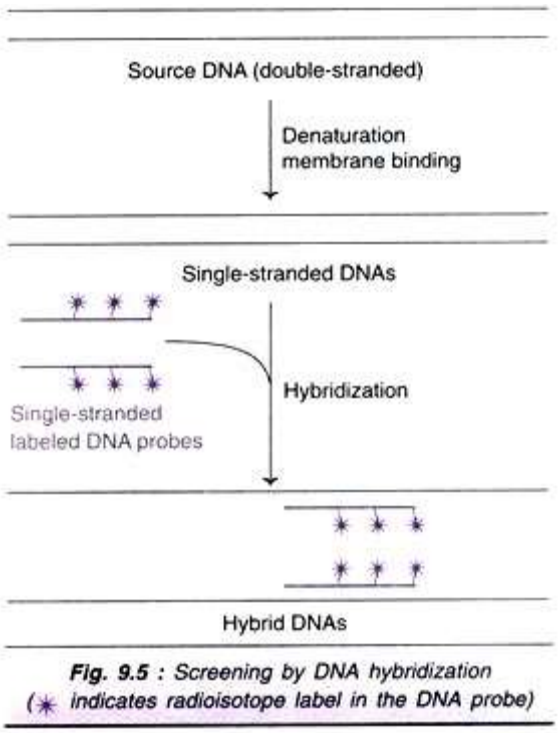


Fig. 9.5 : Screening by DNA hybridization (indicates radioisotope label in the DNA probe)*

Now, the single strands of DNA probe (100-1,000 bp) labeled with radioisotope are added. Hybridization (i.e., base pairing) occurs between the complementary nucleotide sequences of the target DNA and the probe. For a stable base pairing, at least 80% of the bases in the two strands (target DNA and the probe) should be matching. The hybridized DNA can be detected by autoradiography.

DNA Probes:

The DNA probes used for screening purpose can be synthesized in many ways.

Random primer method:

Radioisotope labelled DNA primers can be produced by this technique (Fig. 9.6). The double-stranded DNA containing the sequence needed to serve as a probe is denatured. A mixture of synthetic oligonucleotides, with all possible combinations of bases (A, G, C and T), with a length of 6 nucleotides each serve as primers. Some of these primers with complementary sequences will hybridize with the template DNA. This occurrence is entirely by chance and the probability is reasonably good.

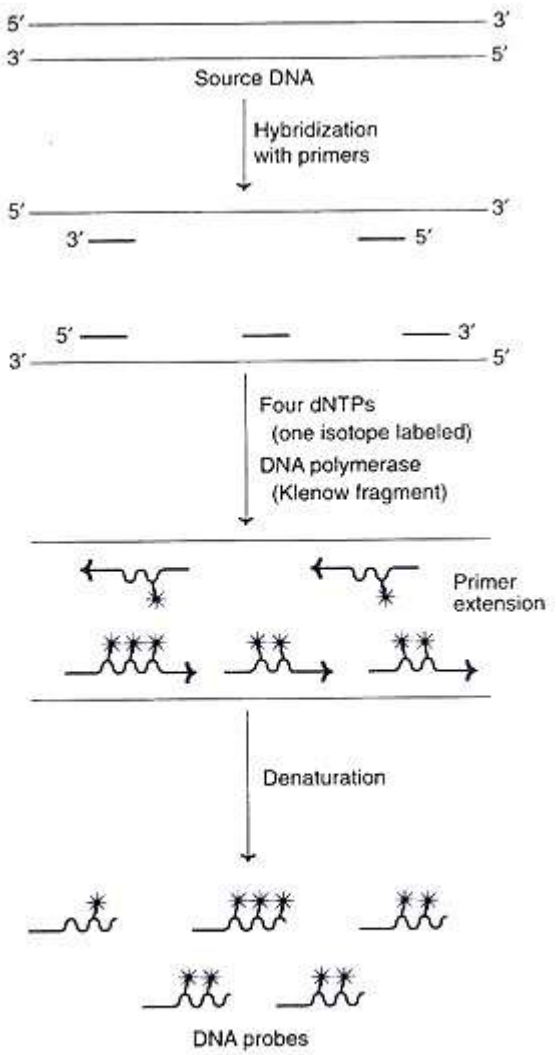


*Fig. 9.6 : Synthesis of radioisotope labeled DNA probes (**Note** : Non-isotopic DNA probes can be prepared by tagging with chemical labels e.g. biotin)*

By the addition of four deoxyribonucleotides (one of them is radiolabelled) and in the presence of the enzyme DNA polymerase of E. coli (Klenow fragment), the primers are extended on the template DNA. Since a radioactive label is used, the newly synthesized DNA fragments are labelled at appropriate places, and these are the DNA probes. A number of labelled DNA probes can be produced from an unlabelled template DNA.

Non-isotopic DNA probes:

For the production of non-isotopic DNA probes, one of the four deoxynucleotides (used for primer extension described above) is tagged with a label (e.g., biotin). The label of the DNA probes can be detected by use of chemical and enzymatic reactions.

2. Screening by Colony Hybridization:

The DNA sequence in the transformed colonies can be detected by hybridization with radioactive DNA probes (sometimes labelled RNA probes can also be used). Colony hybridization technique is also referred to as replica plating by some authors. The technique depicted in Fig. 9.7 is briefly described.

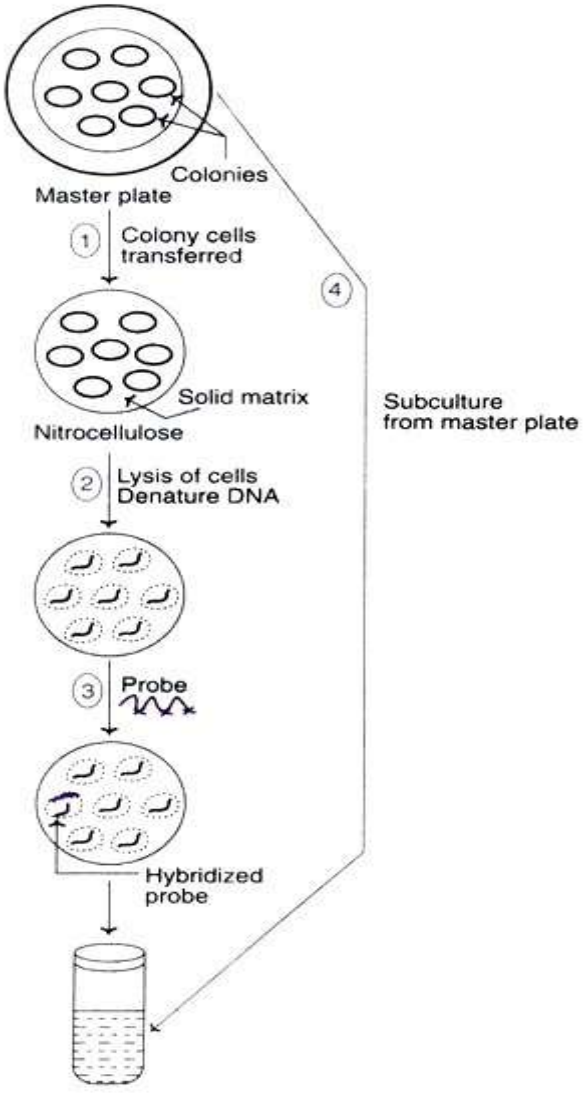


*Fig. 9.7 : Screening by colony hybridization (**Note** : Step 4 is carried out for the colony identified in step 3).*

The transformed cells are grown as colonies on a master plate. Samples of each colony are transferred to a solid matrix such as nitrocellulose or nylon membrane. The transfer is carefully carried out to retain the pattern of the colonies on the master plate. Thus, the nitrocellulose paper contains a photocopy pattern of the master plate colonies. The colony cells are lysed and deproteinized. The DNA is denatured and irreversibly bound to matrix. Now a radiolabelled

DNA probe is added which hybridizes with the complementary target DNA. The non-hybridized probe molecules are washed away. The colony with hybridized probe can be identified on autoradiograph. The cells of this colony (from the master plate) can be isolated and cultured.

Many a times multiple colonies are detected on hybridization by a DNA probe. This is due to overlapping sequences. To identify which colony has the complete sequence of the target gene, data observed from the restriction endonuclease analysis will be helpful.

Modifications of Colony Hybridization Technique:

Several improvements in the colony hybridization technique, described above, have been made in recent years. In the plaque lift technique, nitrocellulose paper is directly applied on the upper surface of master agar plate making a direct contact. By this way, plaques can be lifted and several identical DNA prints can be made from a single plate. This technique increases reliability. More recently, screening of DNA libraries is carried out by automated techniques.

3. Screening by PCR:

Polymerase chain reaction (PCR) is as good as hybridization technique for screening DNA libraries. But adequate information (on the franking sequences of target DNA) must be available to prepare primers for this method. The colonies are maintained in multiwall plates, each well is screened by PCR and the positive wells are identified.

4. Screening by Immunological Assay:

Immunological techniques can be used for the detection of a protein or a polypeptide, synthesized by a gene (through transcription followed by translation). The procedure adopted for immunological assay and hybridization technique (described already) are quite comparable. Screening procedure by immunological assay is depicted in Fig. 9.8, and briefly described hereunder.

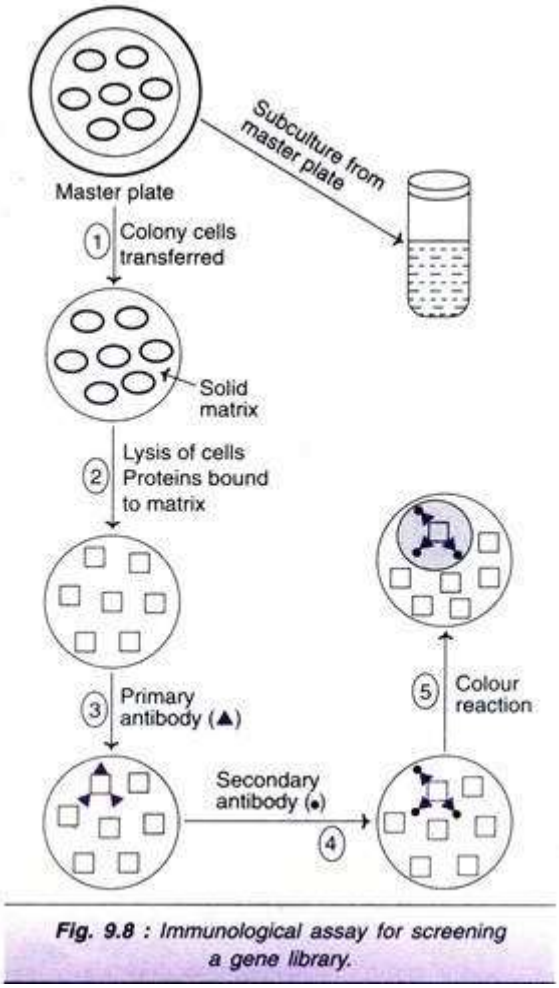


Fig. 9.8 : Immunological assay for screening a gene library.

The cells are grown as colonies on master plates which are transferred to a solid matrix (i.e., nitrocellulose). The colonies are then subjected to lysis and the released proteins bound to the matrix. These proteins are then treated with a primary antibody which specifically binds to the protein (acts as an antigen), encoded by the target DNA. After removing the unbound antibody by washings, a second antibody is added which specifically binds to the first antibody.

Again the unbound antibodies are removed by washings. The second antibody carries an enzyme label (e.g., horse reddish peroxidase or alkaline phosphatase) bound to it. The detection process is so devised that as a colourless substrate it is acted upon by this enzyme, a coloured product is formed. The colonies which give positive result (i.e., coloured spots) are identified. The cells of a specific colony can be sub-cultured from the master plate.

5. Screening by Protein Function:

If the target DNA of the gene library is capable of synthesizing a protein (particularly an enzyme) that is not normally produced by the host cell, the protein activity can be used for screening. A specific substrate is used, and its utilization by a colony of cells indicates the presence of an enzyme that .acts on the substrate. For instance, the genes coding for enzymes α-amylase and β-glucosidase can be identified by this technique.

Genomic and cDNA Libraries

Genomic DNA Library:

A genomic library is a collection of independently isolated vector linked DNA fragments derived from a single organism. It contains at least one copy of every DNA sequence in the genome. An ideal library is one that represents all of the sequences with smallest possible number of clones.

The genomic DNA libraries can be prepared by the complete digestion of the total genomic DNA with a restriction enzyme and the fragments are inserted into a suitable vector like X phages (Fig. 22.11). The drawback of this method is that sometimes the sequence of interest may contain multiple restriction sites, so digestion with RE results into two or more pieces.

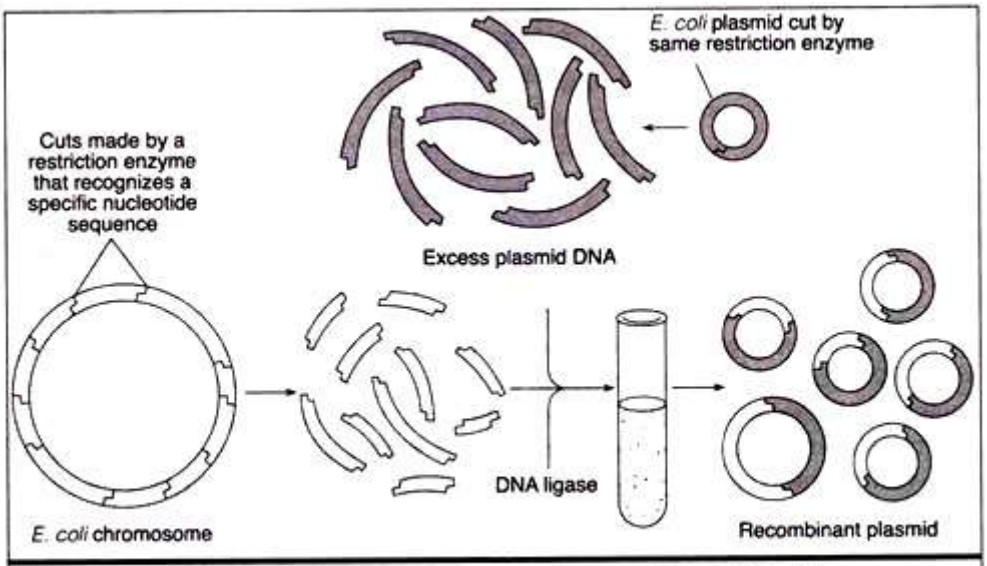


Fig. 22.11: Construction of a genomic library using recombinant DNA techninque

In this method the eukaryotic DNA is broken up into smaller fragments, thus an entire library would necessarily contain a large number of phages, and screening of which is very laborious. The problems of this method can be avoided by random shearing of total DNA and cloning of large fragments.

This method ensures that sequences are not excluded from the cloned library simply because of the distribution of restriction sites. In this procedure the randomly fragmented DNA is partially digested with RE which has short recognition sites. The fragments of desired size are collected through agarose gel electrophoresis, so the population of overlapping fragments that are close to random can be cloned directly.

Constructing Gene Libraries:

a. Digesting Genomic DNA Molecules:

After genomic DNA has been isolated and purified, it is digested with restriction endonucleases. These enzymes are the key to molecular cloning because of the specificity they have for particular DNA sequences. It is important to note that every copy of a given DNA molecule from a specific organism will give the same set of fragments when digested with a particular enzyme.

DNA from different organisms will, in general, give different sets of fragments when treated with the same enzyme. By digesting complex genomic DNA from an organism it is possible to reproducibly divide its genome into a large number of small fragments, each approximately the size of a single gene. Some enzymes cut straight across the DNA to give flush or blunt ends.
Other restriction enzymes make staggered single-strand cuts, producing short single-stranded projections at each end of the digested DNA. These ends are not only identical but complementary and will base-pair with each other; they are, therefore, known as cohesive or sticky ends. In addition, the 5′-end projection of the DNA always retains the phosphate groups.
Over 500 restriction enzymes, recognizing more than 200 different sites, have been characterized. The choice of which enzyme to use depends on a number of factors. For example, the recognition sequence of 6 bp will occur, on average, every 4096 (4^6) bases, assuming a random sequence of each of the four bases.
This means that digesting genomic DNA with EcoRI, which recognizes the sequence 5′-GAATTC-3′, will produce fragments each of which is, on average, just over 4 kb. Enzymes with 8 bp recognition sequences produce much longer fragments. Therefore, very large genomes, such as human DNA, are usually digested with enzymes that produce long DNA fragments. This makes subsequent steps more manageable, since a smaller number of those fragments need to be cloned and subsequently analysed.

b. Ligating DNA Molecules:

The DNA products resulting from restriction digestion to form sticky ends may be joined to any other DNA fragments treated with the same restriction enzyme. Thus, when the two sets of fragments are mixed; base-pairing between sticky ends will result in the annealing of fragments that were derived from different starting DNA. There will, of course, also be pairing of fragments derived from the same starting DNA molecules, termed re-annealing.
All these pairing are transient, owing to the weakness of hydrogen bonding between the few bases in the sticky ends, but they can be stabilized by use of an enzyme, DNA ligase, in a process termed ligation. This enzyme, usually isolated from bacteriophage T4 and called T4 DNA ligase, forms a covalent bond between the 5′- phosphate at the end of one strand and the 3′-hydroxyl of the adjacent strand.
The reaction which is ATP dependent is often carried out at 10°C to lower the kinetic energy of the molecules, and so reduce the chances of base-paired sticky ends parting before they have been stabilized by ligation. However, long reaction times are needed to compensate for the low activity of DNA ligase in the cold. It is also possible to join blunt ends of DNA molecules, although the efficiency of this reaction is much lower than in sticky-ended ligations.
Since ligation reconstructs the site of cleavage, recombinant molecules produced by ligation of sticky ends can be cleaved again at the ‘joins’, using the same restriction enzyme that was used to generate the fragments initially. In order to propagate digested DNA from an organism it is necessary to join or ligate that DNA with a specialized DNA carrier molecule termed a vector. Each DNA fragment is inserted by ligation into vector DNA molecule, which allows the whole recombinant DNA to then be replicated indefinitely within microbial cells. In this way a DNA fragment can be cloned to provide sufficient material for further detailed analysis or for further manipulations. Thus, all of the DNA extracted from an organism and digested with a restriction enzyme will result in a collection of clones. This collection of clones is known as a gene library.

Genomic Libraries:

Any particular gene constitutes only a small part of an organism's genome. For example, if the organism is a mammal whose entire genome encompasses some 106 kbp and the gene is 10 kbp, then the gene represents only 0.001% of the total nuclear DNA. It is impractical to attempt to recover such rare sequences directly from isolated nuclear DNA because of the overwhelming amount of extraneous DNA sequences.

Instead, a genomic library is prepared by isolating total DNA from the organism, digesting it into fragments of suitable size, and cloning the fragments into an appropriate vector. This approach is called shotgun cloning because the strategy has no way of targeting a particular gene but instead seeks to clone all the genes of the organism at one time.

The intent is that at least one recombinant clone will contain at least part of the gene of interest. This can be achieved by partial restriction digestion with an enzyme that recognizes tetra nucleotide sequences. Complete digestion with such an enzyme would produce a large number of very short fragments, but, if the enzyme is allowed to cleave only a few of its potential restriction sites before the reaction is stopped, each DNA molecule will be cut into relatively large fragments.

Average fragment size will depend on the relative concentrations of DNA and restriction enzyme and, in particular, on the conditions and durations of incubation. It is also possible to produce fragments of DNA by physical shearing, although the ends of the fragments may need to be repaired to make them flush ended. This is achieved by using a modified DNA polymerase termed Klenow polymerase.

This is prepared by cleavage of DNA polymerase with subtilizing, giving a large enzyme fragment which has no 5'→3′ exonuclease activity, but which still acts as 5'→3′ polymerase. Using the appropriate dNTPs, this will fill in any recessed 3′ ends on the sheared DNA. The mixture of DNA fragments is then ligated with a vector, and subsequently cloned. If enough clones are produced there will be a very high chance that any particular DNA fragment, such as a gene, will be present in at least one of the clones. To keep the number of clones to a manageable size, fragments about 10 kb in length are needed for prokaryotic libraries, but the length must be increased to about 40 kb for mammalian libraries. Genomic libraries have been prepared from hundreds of different species. Many clones must be created to be confident that the genomic library contains the gene of interest. The probability, P, that some number of clones, N, contains a particular fragment representing a fraction, f, of the genome is

$$P = 1 - (1 - f)^N.$$

$$\text{Thus, } N = \text{In } (1 - P)/ \text{ In } (1 - f).$$

For example, if the library consists of 10 kbp fragments of the E. coli genome (4640 kbp total), over 2000 individual clones must be screened to have a 99% probability (P = 0.99) of finding a particular fragment. Since/ =10/4640 = 0.0022 and P – 0.99, N = 2093. For a 99% probability of finding a particular sequence within the 3×10^6 kbp human genome, N would equal almost 1.4 million if the cloned fragments averaged 10 kbp in size. The need for cloning vectors capable of carrying very large DNA inserts becomes obvious from these numbers.

Screening Libraries:

A common method of screening plasmid-based genomic libraries is to carry out a colony hybridization experiment. The protocol is similar for phage-based libraries except that bacteriophage plaques, not bacterial colonies, are screened. In a typical experiment, host bacteria containing either a plasmid based or bacteriophage-based library are plated out on a petri dish and allowed to grow overnight to form colonies (or in the case of phage libraries, plaques) (Fig 4.10).

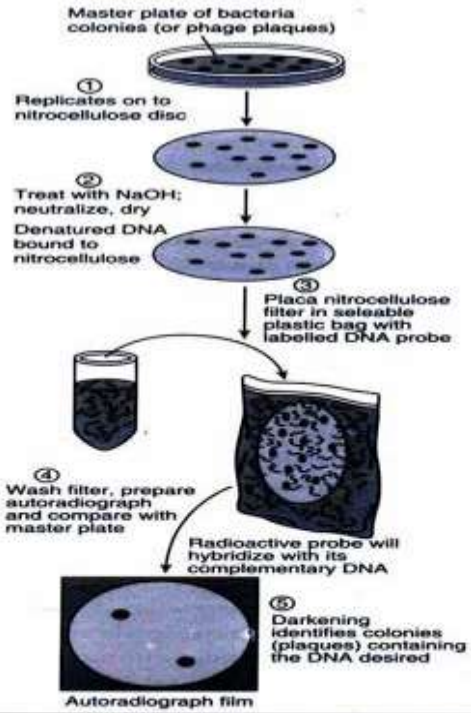


Fig. 4.10: Screening a genomic library by colony hybridization (or plaque hybridization). Host bacteria transformed with a plasmid-based genomic library or infected with a bacteriophage-based genomic library are plated on a petri plate and incubated overnight to allow bacterial colonies (or phage plaques) to form. A replica of the bacterial colonies (or plaques) is then obtained by overlaying the plate with a nitrocellulose disc. (1) Nitrocellulose strongly binds nucleic acids; single-stranded nucleic acids are bound more tightly than double-stranded nucleic acids. (Nylon membranes with similar nucleic acid – and protein-binding properties are also used.) Once the nitrocellulose disc has taken up an impression of the bacterial colonies (or plaques), it is removed and the petri plate is set aside and saved. The disc is treated with 2 M NaOH, neutralized, and dried. (2) NaOH both lyse any bacteria (or phage particles) and dissociates the DNA strands. When the disc is dried, the DNA strands become immobilized on the filter. The dried disc is placed in a sealable plastic bag, and a solution containing heat-denatured (single-stranded), labelled probe is added. (3) The bag is incubated to allow annealing of the probe DNA to any target DNA sequences that might be present on the nitrocellulose. The filter is then washed, dried, and placed on a piece of X-ray film to obtain an autoradiogram. (4) The position of any spots on the X-ray film reveals where the labelled probe has hybridized with target DNA. (5) The location of these spots can be used to recover the genomic clone from the bacteria (or plaques) on the original petri plate.

A replica of the bacterial colonies (or plaques) is then obtained by overlaying the plate with a nitrocellulose disc. The disc is removed, treated with alkali to dissociate bound DNA duplexes into single-stranded DNA, dried, and placed in a sealed bag with labelled probe. If the probe DNA is duplex DNA, it must be denatured by heating at 70°C.

The probe and target DNA complementary sequences must be in a single stranded form if they are to hybridize with one another. Any DNA sequences complementary to probe DNA will be revealed by autoradiography of the nitrocellulose disc. Bacterial colonies (phage plaques) containing clones bearing target DNA are identified on the film and can be recovered from the master plate.

Probes for Southern Hybridization:

Clearly, specific probes are essential reagents if the goal is to identify a particular gene against a background of innumerable DNA sequences. Usually, the probes that are used to screen li-

braries are nucleotide sequences that are complementary to some part of the target gene. To make useful probes requires some information about the gene's nucleotide sequence.
Sometimes such information is available. Alternatively, if the amino acid sequence of the protein encoded by the gene is known, it is possible to work backward through the genetic code to the DNA sequence (Fig. 4.11). Because the genetic code is degenerate (that is, several codons may specify the same amino acid), probes designed by this approach are usually degenerate oligonucleotides about 17 to 50 residues long (such oligonucleotides are so-called 17- to 50- mers).

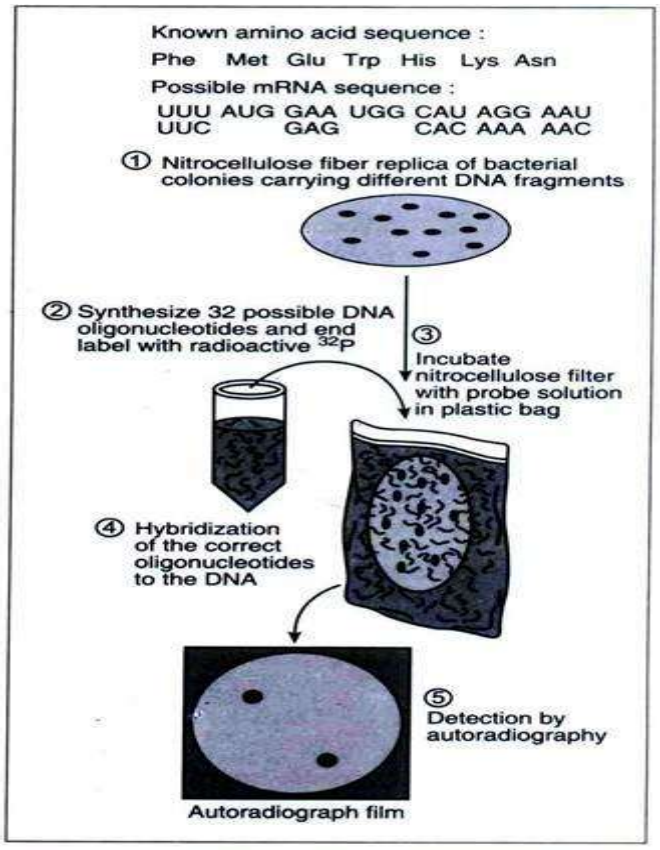


Fig. 4.11: sequence. A radioactively labelled set of DNA (degenerate) oligonucleotides representing all possible mRNA coding sequences is synthesized. (In this case, there are 25 or 32.) The complete mixture is used to probe the genomic library by colony hybridization.

The oligonucleotides are synthesized so that different bases are incorporated at sites where degeneracies occur in the codons. The final preparation thus consists of a mixture of equal-length oligonucleotides whose sequences vary to accommodate the degeneracies. Presumably, one oligonucleotide sequence in the mixture will hybridize with the target gene. These oligonucleotide probes are at least 17-mers because shorter degenerate oligonucleotides might hybridize with sequences unrelated to the target sequence.
A piece of DNA from the corresponding gene in a related organism can also be used as a probe in screening a library for a particular gene. Such probes are termed heterologous probes because they are not derived from the homologous (same) organism. Problems arise if a complete eukaryotic gene is the cloning target; eukaryotic genes can be tens or even hundreds of kilo-base pairs in size.

Genes of this size are fragmented in most cloning procedures. Thus, the DNA identified by the probe may represent a clone that carries only part of the desired gene. However, most cloning strategies are based on a partial digestion of the genomic DNA, a technique that generates an overlapping set of genomic fragments. This being so, DNA segments from the ends of the identified clone can now be used to probe the library for clones carrying DNA sequences that flanked the original isolate in the genome. Repeating this process ultimately yields the complete gene among a subset of overlapping clones.

cDNA Libraries:

cDNAs are DNA molecules copied from mRNA templates. cDNA libraries are constructed by synthesizing cDNA from purified cellular mRNA. These libraries present an alternative strategy for gene isolation, especially eukaryotic genes. Because most eukaryotic mRNAs carry 3′-poly(A) tails, mRNA can be selectively isolated from preparations of total cellular RNA by oligo(dT)-cellulose chromatography (Fig. 4.12). DNA copies of the purified mRNAs are synthesized by first annealing short oligo (dT) chains to the poly(A) tails.

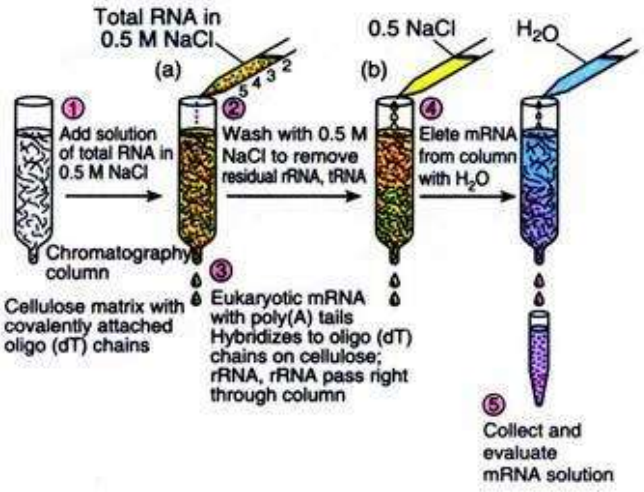


Fig. 4.12: Isolation of eukaryotic mRNA via oligo(dT)-cellulose chromatography. (a) In the presence of 0.5 M NaCl, the poly(A) tails of eukaryotic mRNA anneal with short oligo(dT) chains covalently attached to an insoluble chromatographic matrix such as cellulose. Other RNAs, such as rRNA (green), pass right through the chromatography column. (b) The column is washed with more 0.5 M NaCl to remove residual contaminants. (c) Then the poly(A) mRNA is recovered by washing the column with water because the base pairs formed between the poly(A) tails of the mRNA and the oligo(dT) chains are unstable in solutions of low ionic strength.

These oligo(dT) chains serve as primers for reverse transcriptase-driven synthesis of DNA (Fig. 4.13). (Random oligonucleotides can also be used as primers, with the advantages being less dependency on poly(A) tracts and increased likelihood of creating clones representing the 5′-ends of mRNAs.) Reverse transcriptase is an enzyme that synthesizes a DNA strand, copying RNA as the template. DNA polymerase is then used to copy the DNA strand and form a double-stranded (duplex DNA) molecule.

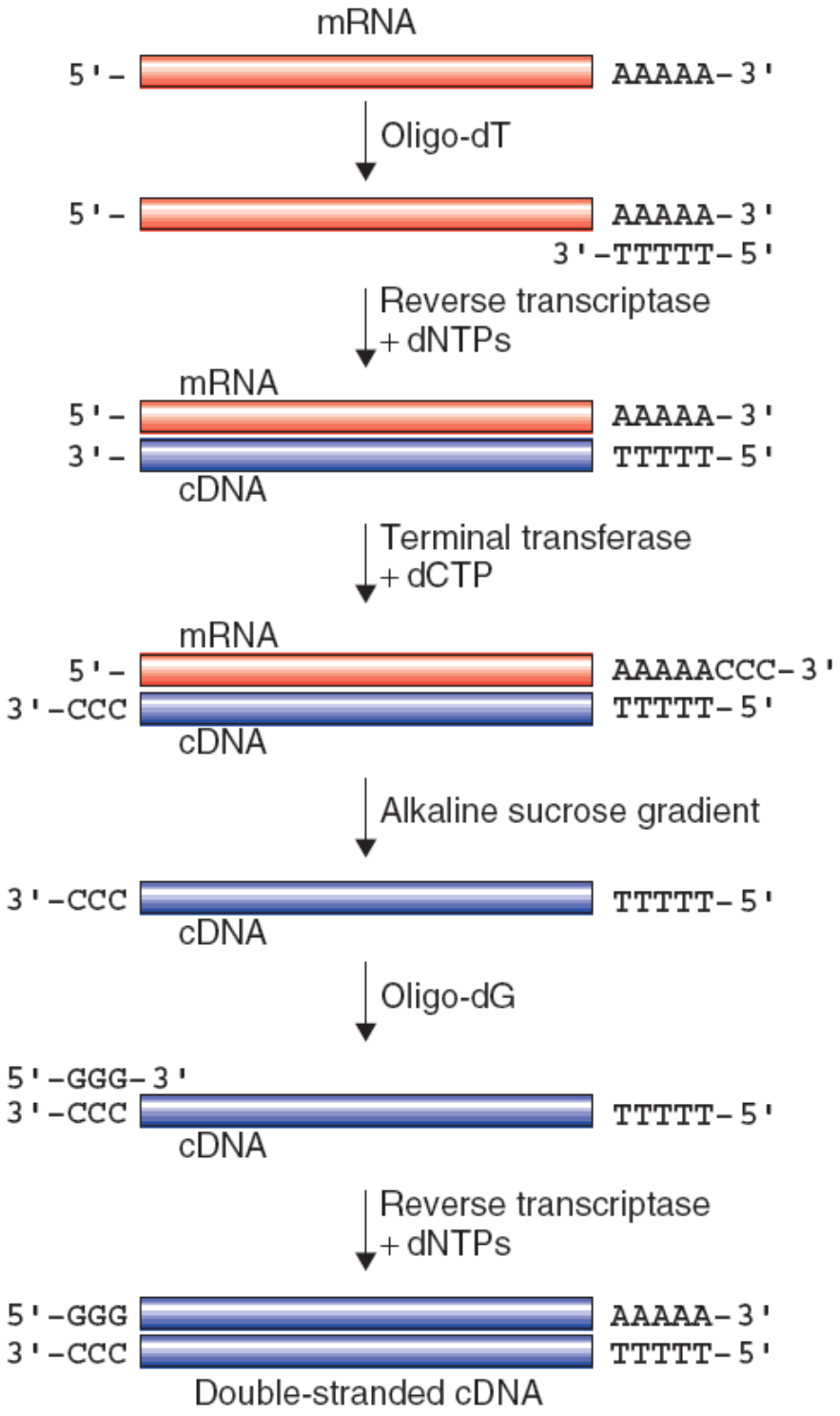


Fig: The construction of a cDNA library.

Ligation of blunt-ended DNA fragments is not as efficient as ligation of sticky ends; therefore, with cDNA molecules additional procedures are undertaken before ligation with cloning vectors. One approach is to add cDNA small, double stranded molecules with one internal site for a restriction endonuclease; these are termed nucleic acid linkers. Numerous linkers are commercially available with internal restriction for many of the most commonly used restriction enzymes.

Linkers are blunt end ligated to cDNA but since they are added much in excess of the cDNA, the ligation process is reasonably successful. Subsequently the linkers are digested with the appropriate restriction enzyme, which provides the sticky ends for efficient ligation to a vector digested with the same enzyme. This process may be made easier by the addition of adaptors rather than linkers, which are identical except that the sticky ends are performed and so there is no need of restriction digestion following ligation.
Therefore, lastly Linkers are added to the DNA duplexes rendered from the mRNA templates, and the cDNA is cloned into a suitable vector. Once a cDNA derived from a particular gene has been identified, the cDNA becomes an effective probe for screening genomic libraries for isolation of the gene itself. Because different cell types in eukaryotic organisms express selected subsets of genes, RNA preparations from cells or tissues in which genes of interest are selectively transcribed are enriched for the desired mRNAs. cDNA libraries prepared from such mRNA are representative of the pattern and extent of gene expression that uniquely define particular kinds of differentiated cells.
cDNA libraries of many normal and diseased human cell types are commercially available, including cDNA libraries of many tumour cells. Comparison of normal and abnormal cDNA libraries, in conjunction with two dimensional gel electrophoretic analysis of the proteins produced in normal and abnormal cells is a promising new strategy in clinical medicine to understand disease mechanisms.

Phage and phasmid display

In phage display, a segment of foreign DNA is inserted into either a phagemid or an infectious filamentous phage genome and expressed as a fusion product with a phage coat protein. It is a very powerful technique for selecting and engineering polypeptides with novel functions. The technique was developed first for the E. coli phage M13 but has since been extended to other phages such as T4 and lambda. The M13 phage particle consists of a single-stranded DNA molecule surrounded by a coat consisting of several thousand copies of the major coat protein, P8. At one end of the particle are five copies each of the two minor coat proteins P9 and P7 and at the other end five copies each of P3 and P6. In early examples of phage display, a random DNA cassette was inserted into either the P3 or the P8 gene at the junction between the signal sequence and the native peptide. E. coli transfected with the recombinant DNA molecules secreted phage particles that displayed on their surface the amino acids encoded by the foreign DNA. Particular phage displaying peptide motifs with, for example, antibody binding properties were isolated by affinity chromatography. Several rounds of affinity chromatography and phage propagation can be used to further enrich for phage with the desired binding characteristics. In this way, millions of random peptides have been screened for their ability to bind to an anti-peptide antibody or to streptavidin and variants of human growth hormone with improved affinity and receptor specificity have been isolated. One disadvantage of the original method of phage display is that polypeptide inserts greater than 10 residues compromise coat-protein function and so cannot be efficiently displayed. This problem can be solved by the use of phagemid display. In this system, the starting-point is a plasmid carrying a single copy of the P3 or P8 gene from M13 plus the M13 ori sequence. As before, the random DNA sequence is inserted into the P3 or P8 gene downstream from the signal peptide-cleavage site and the construct transformed into E. coli. Phage particles displaying the amino acid sequences encoded by the DNA insert are obtained by superinfecting the transformed cells with helper phage. The resulting phage particles are phenotypically mixed and their surfaces are a

mosaic of normal coat protein and fusion protein. Specialized phagemid display vectors have been developed for particular purposes. For example, phagemids have been constructed that have an amber (chain-terminating) codon immediately downstream from the foreign DNA insert and upstream from the body of P3 or P8. When the recombinant phagemid is transformed into non-suppressing strains of E. coli, the protein encoded by the foreign DNA terminates at the amber codon and is secreted into the medium. However, if the phagemid is transformed into cells carrying an amber suppressor, the entire fusion protein is synthesized and displayed on the surface of the secreted phage particles. Other studies have shown that proteins can be displayed as fusions to the carboxy terminus of P3, P6 and P8. Although amino-terminal display formats are likely to dominate established applications, carboxy-terminal display permits constructs that are unsuited to amino terminal display.

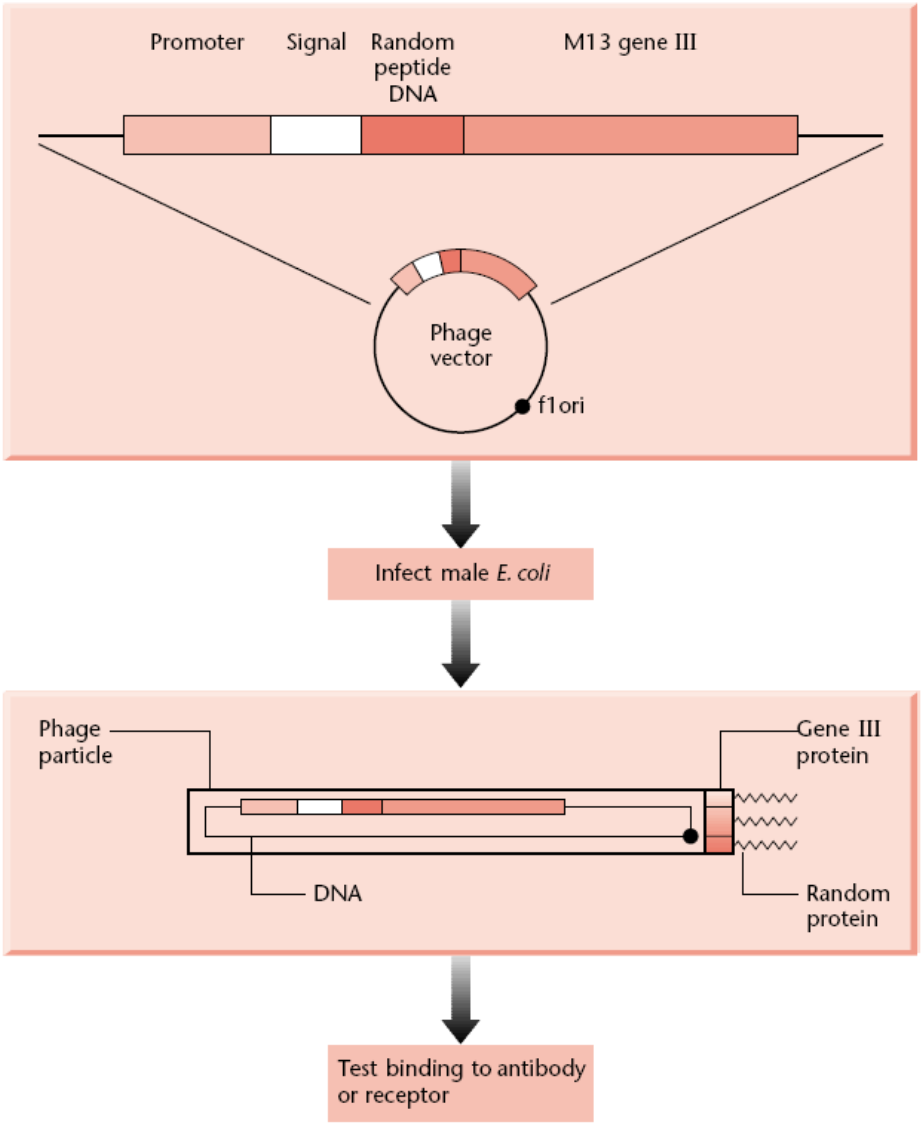


Fig: The principle of phage display of random peptides

Probable Questions:

1. Define cloning vector? How it differ from expression vector?
2. What are the characteristics of a ideal vector.
3. What are the advantages of plasmid as cloning vector.
4. differentiate insertion type and replacement type cloning phage vector.
5. Write down the advantages of cosmid vector.
6. What is YAC. State its utility as cloning vector.
7. What is BAC. State its utility as cloning vector.
8. How electroporation is used in transformation in E. coli?
9. How liposome mediated gene transfer occurs in E.coli ?
10. How colony hybridization technique helps in screening of transformed bacteria.
11. How immunological assay help in screening of transformed cells ?
12. How genomic library is constructed?
13. What is cDNA? How cDNA library is made?
14. What is phage display? Write its utility.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
9. Primrose SB and Twyman RM. (2006) Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
10. Sambrook J and Russell D. (2001) Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Pres

Unit-VI

Manipulating genes in animals: gene transfer to animal cells, genetic manipulation of animals, transgenic technology, application of recombinant DNA technology; genetically modified organisms: gene knockouts, mouse disease models, gene silencing, gene therapy, somatic and germ- line therapy

Objective: In this unit you will learn about various techniques which are used for introduction of foreign genes into animal cells. You will also have an idea on GMO and their uses. Gene silencing methods including knock out mice and gene therapy methods will also be discussed in this unit.

Gene transfer methods in Animal cells:
There are several methods available by which foreign gene (transgene) can be transferred into animal cells.

Method - 1. Calcium Chloride ($CaCl_2$) Mediated DNA Transfer:
This is used for the transformation of prokaryotic host cells.

Principle:

In the process of transformation all bacterial cells cannot uptake the exogenous DNA molecule. Those who are capable to take are called competent cells. So our aim in this step is to make bacterial cells more competent so that the possibility of transferring of the recombinant DNA into the host cell increases to a higher fold. $CaCl_2$ makes the cell wall of the bacteria more permeable to the exogenous DNA and thus increases the competence of the host cell.

Procedure:

Growing E. Coli cells are isolated and suspended in 50 mM $CaCl_2$ at a concentration of 108-1010 cells/ml. The cells may be incubated for 12- 24 hr. to increase the frequency of transformation. The recombinant DNA is then added.
Efficient transformation takes only a few minutes and the cells are plated on a suitable medium for the selection of transformed clones. The frequency of transformed cells is 106-107 per mg of plasmid DNA; this is about one transformation per 10,000 plasmid molecules.
The transformed cells are suitably diluted and spread thinly on a suitable medium so that each cell is well separated and produces a separate colony. Generally, the medium is so designed that it permits only the transformed cells to divide and produce colonies. This frequency can be further improved by using special E. Coli strains, e.g., SK1590, SK1592, X1766, etc.

Method - 2. Rubidium Chloride Mediated DNA Transfer:

The rubidium chloride method is a variant of the calcium chloride method that offers somewhat higher competency. The process followed is same as before but just the $CaCl_2$is replaced with $RbCl_2$. This is also used in the transformation of the prokaryotic host cell.

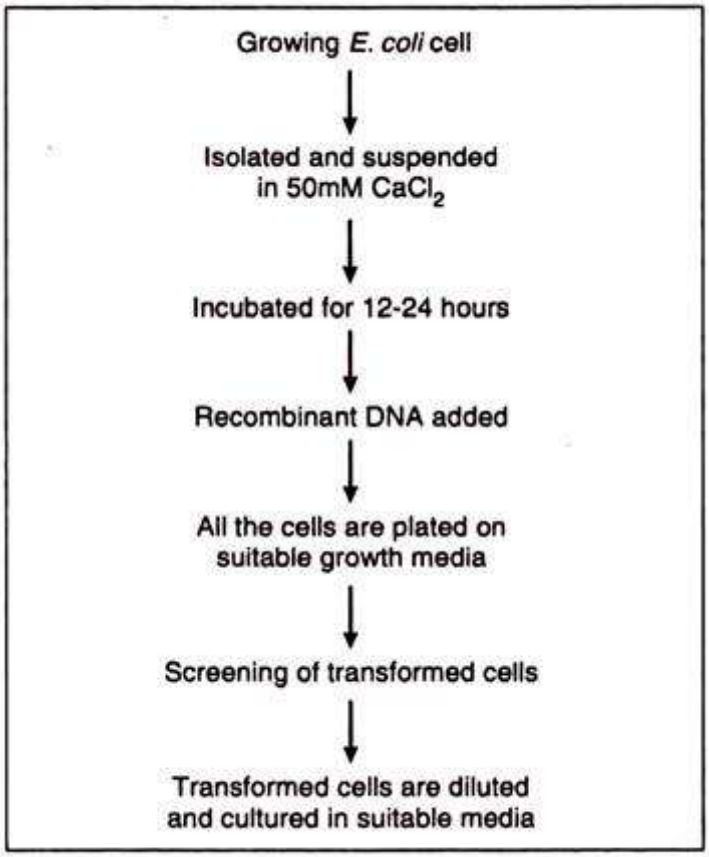


Fig. 5.5: Calcium chloride ($CaCl_2$) mediated DNA transfer

Method – 3: Electroporation:

Electroporation or electro-permeabilization is the process of applying electrical field to a living cell for a brief duration of time in order to create microscopic pores in the plasma membrane called electro-pores. This technique is used for transferring the recombinant DNA molecule into wide range of hosts starting from bacteria to plant (plant protoplasts) and animal cells.

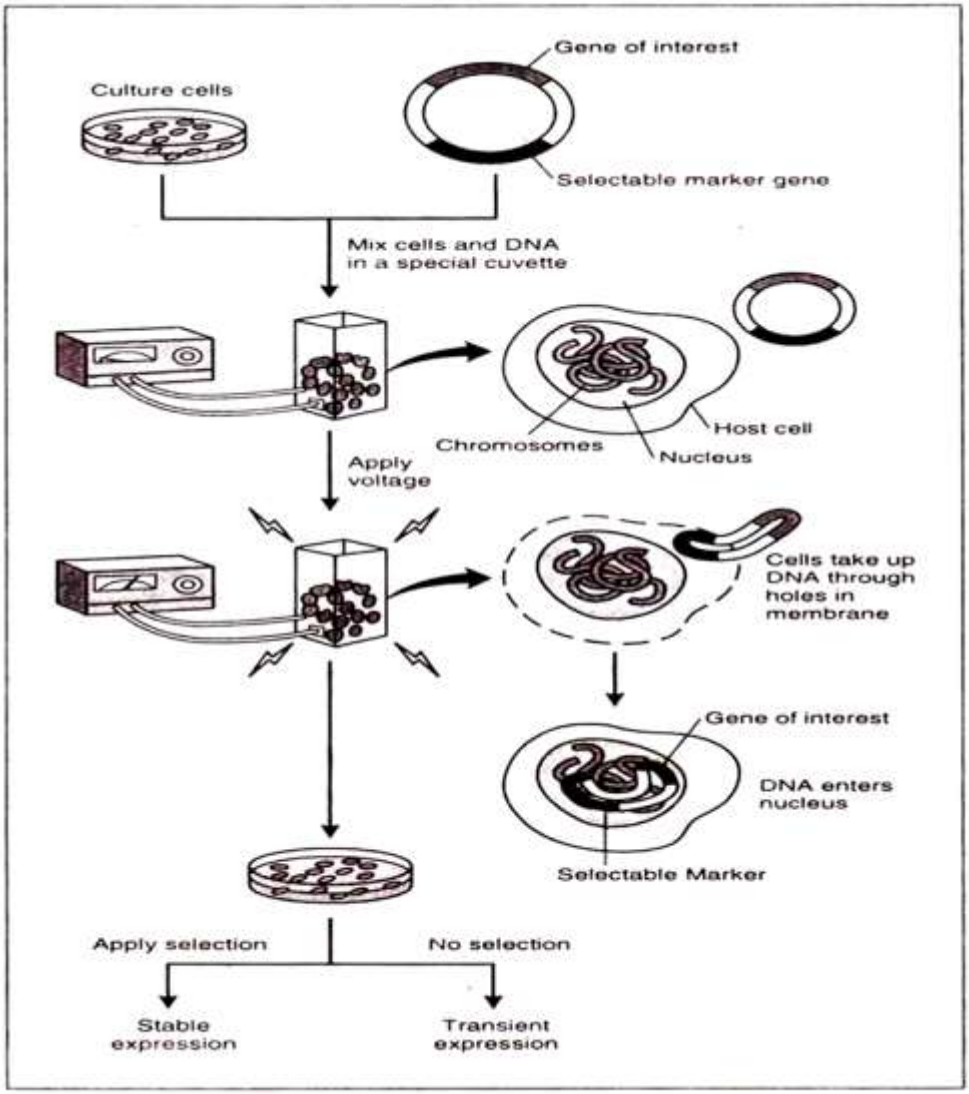


Fig. 5.6: Gene transfer by electroporation

Principle:

The phospholipid molecules of the plasma membrane are not static. When we apply electric field to them their kinetic energy increases resulting in the increase in the membrane permeability at certain points. This is exactly where we see the formation of electro-pores. The recombinant DNA can pass through these transient pores before they close.

Procedure:

In this process cells are mixed with the recombinant DNA and the mixture is placed in a small chamber with electrodes connected to a specialized power supply. Then a brief electric impulse is discharged across the electrodes, which makes pores (holes) in the plasma membrane.
These pores remain for some time and are again resealed themselves. Recombinant DNA enters the cell which are removed and plated in fresh selective medium. The process of selection is then applied to isolate cells carrying recombinant DNA.

Method # 4. Liposome Encapsulation (Lipofection):

This technique is found very successful in the transfection of plant protoplasts and animal host cells.

Principle:

Liposomes are microscopic vesicles developed in a laboratory environment. Each liposome is a spherical ball like structure made up of phospholipid bilayers with a hollow central space, allowing liposomes to interact directly with cells.

A liposome can fuse with the cell membrane of the taken host cell and can deliver its content to it. The recombinant DNA enclosed in the liposome vesicles penetrates into the protoplast of the host cell.

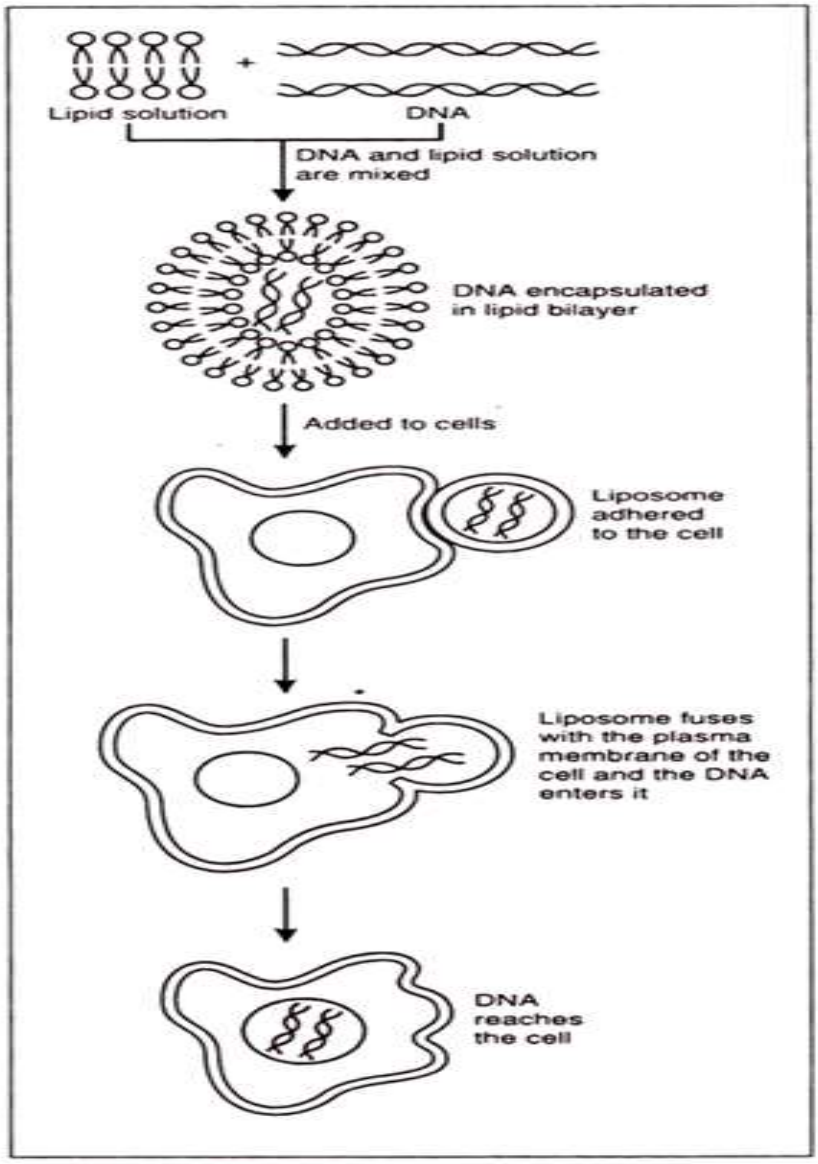


Fig. 5.7: Lipofection

Procedure:

In this technique the recombinant DNA, which is negatively charged at a near neutral pH because of its phosphodiester backbone, is mixed with the lipid molecules with positively

charged (cationic) head groups. The lipid molecules form a bilayer around the recombinant DNA molecules. This results in the formation of liposomes which are further mixed with the host cells. Most eukaryotic cells are negatively charged at their surface, so the positively charged liposomes interact with the cells. Cells take up the lipid-recombinant DNA complexes, and some of the transfected DNA enters the nucleus.

Method -5. Microinjection:

This is the direct introduction of the recombinant DNA into the host cell. This technique has been used successfully with both plan and animal cells. In this procedure the cell is held on a glass capillary by gentle suction.

The microinjection needle is made by drawing out a heated glass capillary to a fine point. Using a micromanipulator (a mechanical device for fine control of the capillary) the needle has been inserted into the nucleus of the host cell.

One obvious disadvantage is that this technique is labour-intensive and not suitable for primary cloning procedures where large numbers of recombinants are required. However, in certain specialised cases it is an excellent method for targeting DNA delivery once a suitable re-combinant has been identified and developed to the point where microinjection is feasible.

Method # 6. Biolistic Particle Delivery System:

A gene gun or a biolistic particle delivery system is a device which can directly bombard small particles coated with the recombinant DNA on the nucleus of the target cell. This technique is often simply referred to as bio-ballistics or biolistics and has been successfully used in the transfection of both plant and animal cells.

In this technique the recombinant DNA is coated with microscopic tungsten particles known as micro-projectiles, which are then accelerated on a macro-projectile by firing a gunpowder charge or by using compressed gas to drive the macro-projectile.

At one end of the ‘gun’ there is a small aperture that stops the macro-projectile but allows the micro-projectiles to pass through. When directed at cells, these micro-projectiles carry the DNA into the cell and, in some cases, stable transformation will occur.

Method # 7. Calcium Phosphate Co-Precipitation:

This technique is used for the transfection of plant and mostly animal cells. The recombinant DNA is mixed with calcium chloride in a phosphate buffer at neutral pH. This results in the formation of recombinant DNA-calcium phosphate complex which appears as a thin precipitate. This precipitate is then added to the host cell.

The precipitate is taken up by the cell by the process of phagocytosis. The recombinant DNA enters the nucleus and integrates into the host’s genome. The transfection efficiency can be increased by exposing the host cell to 10-20% glycerol or Dimethyl sulfoxide (DMSO).

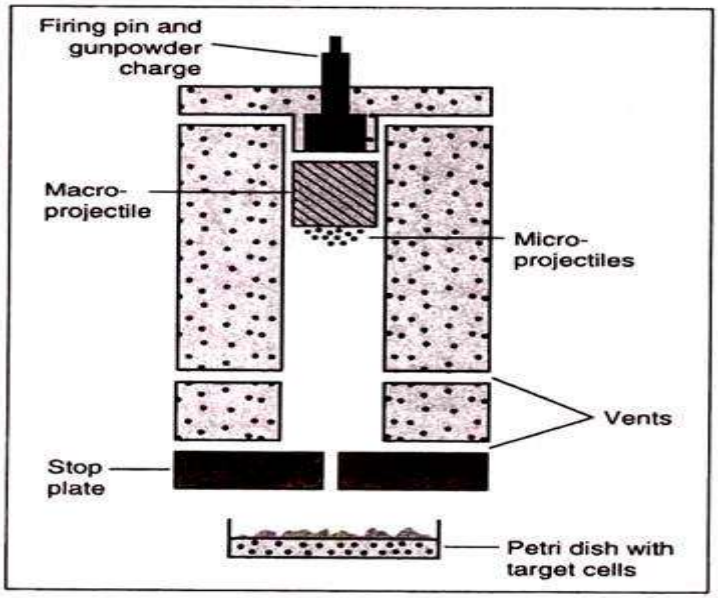


Fig. 5.9: Biolistic particle delivery system

Method - 8. Sonoporation:

Sonoporation, or cellular sonication, is the use of sound (typically ultrasonic frequencies) for the transfer of recombinant DNA into the target host cell. This process has been successfully used in a wide range of host cells starting from bacteria to plant and animal cells.
This employs the acoustic waves to increase the permeability of the plasma membrane. Taking the advantage of this situation the recombinant DNA enters the host cell.

Method - 9. Optical Transfection:

Optical Transfection is the process of introducing nucleic acids into cells using light. This has been successful in transfecting animal cells. In this technique the plasma membrane of the host cell is exposed to the highly focused laser beam for a small amount of time (typically tens of milliseconds to seconds), generating a transient pore on the membrane called photo-pore. Through the photo-pore the recombinant DNA can enter the host cell.

Method - 10. Impalefection:

Impalefection is a method of gene delivery using Nano materials, such as carbon Nano fibres, carbon nanotubes, nanowires, etc. This technique is used for the transfection of plant and animal cells. In this technique needle-like nanostructures are synthesized perpendicularly to the surface of a substrate.Recombinant DNA is attached to the nanostructure surface. A chip with arrays of these needles is then pressed against cells or tissue.

Method - 11. Magnetofection:

Magnetofection, or Magnet assisted transfection is a method, which uses magnetic force to deliver recombinant DNA into target host cells. Nucleic acids are first associated with magnetic nanoparticles. Then, application of magnetic force drives the nucleic acid particle complexes towards and into the target host cells, where the cargo is released. This has been successfully used to transfect the plant and animal cells.

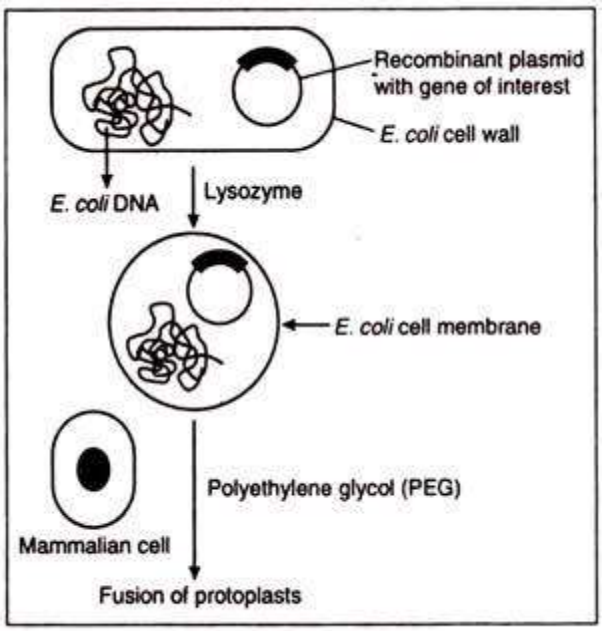


Fig. 5.10: Gene transfer by protoplast fusion

Method - 12. Protoplast Fusion:

This technique is used for introducing gene of interest into plant and animal cells. In this technique first we transfer the recombinant DNA into a bacterial cell then dissolve its cell wall by treating it with lysozyme. After this we fuse the host protoplast with the bacterial cell (lacking cell wall) by the help of polyethylene glycol (PEG). The transfected cells are then selected by suitable methods.

Method - 13. Virus Mediated Gene Transfer:

In other way the gene can be packed into a virus and allow it to infect the host cell without harming it in any way. This method can be used both for the transformation of prokaryotic host cell as well as transfection of eukaryotic host cells. In the case of bacterial host cells the recombinant DNA can be packed into the empty head of a specially designed bacteriophage (e.g., lambda phage) and allow the virion to infect the host cell.

Similarly, while transfecting the plant host cells we can follow the similar strategy by using plant viruses like Caulimo virus and Gemini virus. In the case of animals, retrovirus infection of embryos has been used for the production of transgenic mice.

This virus has been found to be an efficient vector system for animals. The virus carrying the gene of interest transfers it into the genome of embryonic cells leading to its integration and production of transgenic animals.

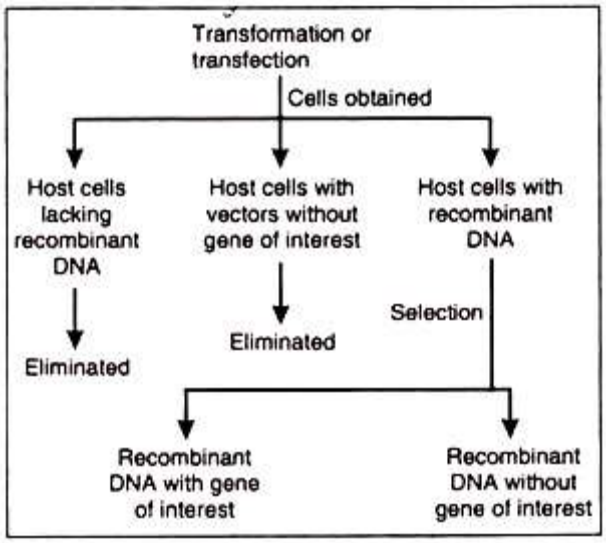


Fig. 5.11: The necessity of selection procedure

Application of Recombinant DNA Technology:

Major applications of recombinant DNA technology are:

1. Medical Diagnosis of Disease
2. Gene Therapy
3. Production of Vaccines through rDNA Technology
4. Cloning
5. Genetically Modified Crops
6. Sustainable Agriculture.

Application - 1. Medical Diagnosis of Disease:

rDNA technology acts as a tool to diagnose the diseases. This involves the construction of probes (short, single strands of radioactive or fluorescent DNA, used to identify the complementary DNA). These probes are used to identify the infectious agents, such as Salmonella (food poisoning), Staphylococcus (pus), HIV, hepatitis virus, etc. With the help of this technique the infected child can also be identified. This can be done by testing the DNA of prospective parents for any genetic disorder, i.e. they are not carrier of a disorder.

Few examples of this technique are as follows:

a. Phenylketonuria:

In this, phenylalanine fails to get converted into tyrosine. This causes disturbances in metabolism resulting in mental retardation. It is possible to cure this disease by using rDNA technique in early periods of pregnancy.

b. Thalassemia Genes:

In this, syntheses of alpha and beta globin chains are reduced and the excess chains precipitate and cause haemolytic anaemia and spleen enlargement. Human globin genes have been identified and sequenced. Alpha and beta globin genes are closely linked. Human globin gene has also been developed and cloned. Still lot of work needs to be done to cure this disease.

c. Haemophilia Gene:
It is a sex linked disease in human where blood clotting does not take place normally due to the absence of clotting factor VIII C. By using gene cloning technique, the clotting factor VIII C gene was cloned to express in mammalian cell lines and produce the protein VIII C responsible for blood clotting.

Application - 2. Gene Therapy:
Gene therapy means to change a faulty gene with a normal, healthy gene. Gene therapy can be used to correct a rare disease, like sickle cell anaemia, which is caused by single mutation and killer diseases such as Severe Combined Immuno Deficiency (SCID). Gene therapy is used to produce recombinant therapeutic bio chemicals such as insulin, somatotropin, somatostatin, interferon, human blood clotting factor VIII, etc.
Several protocols have been developed for expression and introduction of genes in humans, but the clinical efficiency has to be demonstrated conclusively. Success of gene therapy is dependent on the development of better gene transfer vector for sustained, long-term expression of foreign gene as well as better understanding of gene physiology of human disease.

There are two gene transfer strategies:
a. The in vivo approach which involves introduction of genes directly into the target organ of an individual. This is done in patients, therefore called as patient therapy.

b. Ex vivo approach where the cells are isolated for gene transfer in vitro followed by transplantation of genetically modified cells back into the patients.

Application - 3. Production of Vaccines through rDNA Technology:
Human Insulin:
For this the gene of interest is picked up from a human cell. Plasmid from E. coli is taken and by using restriction enzyme it is cut to create sticky ends. Now the gene of interest (insulin gene) and plasmid are joined by DNA ligase. This is now known as rDNA. This rDNA is now inserted in the plasmid free E.coli. Multiplication of rDNA starts growth in medium. Clones of genetically engineered bacteria are used to extract recombinant insulin. In the same way by using hepatitis B virus and Agrobacterium tumefaciens, hepatitis B vaccine and edible vaccine can be generated.

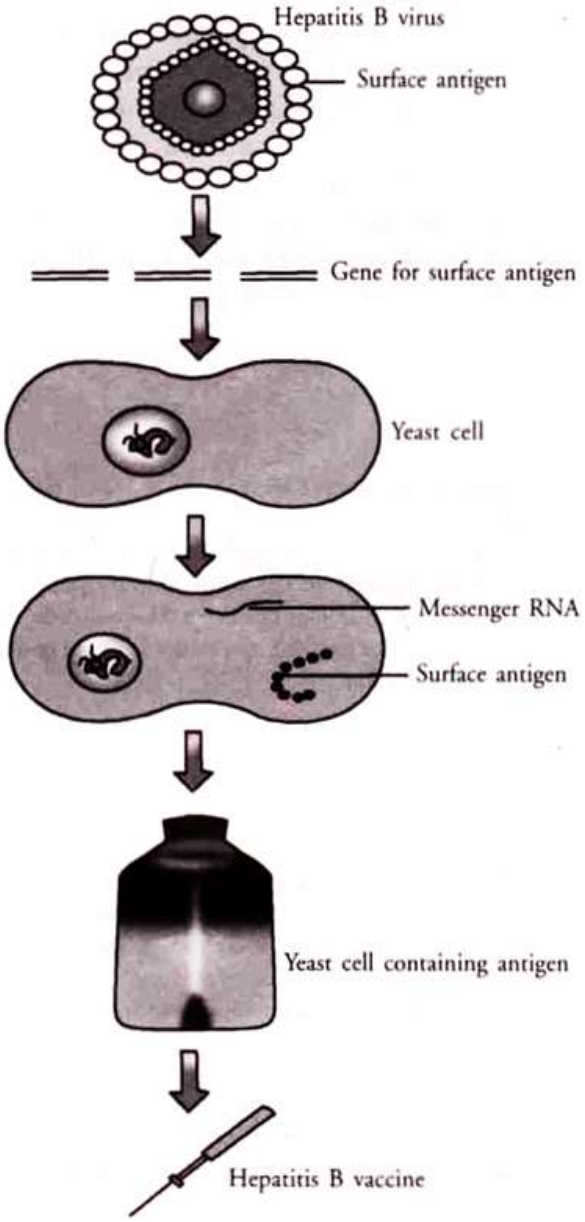


Fig. 2 Formation of injectable hepatitis-B vaccine.

Application - 4. Cloning:

Cloning means to create a carbon copy or identical copy of single parent. This word is related to only the living world and not to non-living world, where we can find thousands of copies of one object like number of photocopies of the same document. In nature asexually reproducing organisms produce clones.

For example, amoeba reproducing by binary fission produce two daughter amoebae which are clones. In human beings, monozygotic, identical twins are clones. They are the result of separation of the two cells of zygote which are in double cell stage. The most famous example of cloning is Dolly sheep.

A. Microbial Cloning:

Once the microbial cells are modified or genetically altered they are cloned on a growth medium. In a few days there are millions of clones generated. Each one is the copy of a single parent. Table 2 shows the genetically modified microbes and their applications.

Table 2 Applications of genetically modified microbes.

Microbes	Applications
Escherichia coli (gut bacterium)	Production of human insulin, human growth factor, interferons, interleukin and so on
Bacillus thuringiensis (soil bacterium)	Production of endotoxin (Bt toxin), highly potent, safe and biodegradable insectide for plant protection
Rhizobium meliloti (bacterium)	Nitrogen fixation by incorporating *'nif'* gene in cereal crops
Pseudomonas fluorescence (bacterium)	Prevention of frost damage to the plants (e.g. strawberries) on which it grows
Pseudomonas putida (bacterium)	Scavenging of oil spills by digesting hydrocarbons of crude oil
Bacterial strains capable of accumulating heavy metal	Bioremediation (cleaning of pollutants in the environment)
Trichoderma (fungus)	Production of enzyme chitinases for biocontrol of fungal diseases in plants

B. Cell Cloning:

This technique is based on the fact that certain cells are totipotent, i.e. they are not differentiated. This phenomenon is seen both in plants and animals. When seen in plants it is called totipotency and when seen in animals it is known as pluriopotency. Almost all the plants show totipotency, whereas in animals pluriopotency is seen in fertilised egg and stem cells in blastocyst. Cells showing pluriopotency can be differentiated into nerve cells, kidney cells and even heart cells.

C. Plant Cloning:

The growth areas of plant, i.e. root and shoot tip are used in plant cloning. This is used to multiply those plants which are agronomically (crop plants) important. Plants which are useful to horticulturist (orchids, gladiolies, etc.) are multiplied at a very fast pace. By gene manipulation we can have drought, disease, insect and pest resistant varieties. We can also have herbicide tolerant variety. Genetically modified food can also be produced like Vitamin A rich rice (Golden Rice), Lysin rich pulse, etc.

Application - 5. Genetically Modified Crops:

A crop which bears a foreign gene of desired function of other organism and expresses itself is called genetically modified crop (GM crop) or transgenic crop. In the last 20 years, considerable progress has been made on isolation, characterisation and introduction of novel genes into plants. In the year 2002, transgenic plants were cultivated on around 587 million hectare land in the world. The number of farmers involved in this was 5.5 million. Transgenic crop plants have many beneficial traits such as pest and insect resistance, weed control, improved oil quality, herbicide tolerance, delayed fruit ripening, etc.

Two main advantages of transgenic crops are:
a. Any gene can be transferred from any organism
b. Change in genotype can be controlled as only the desired gene is introduced.
In contrary to this, when conventional method like hybridisation is used only those genes can be used which are found in such species and along with desirable change undesirable genes are also added.

The effects of introduction of foreign gene of interest are as follows:
a. Existing biosynthetic pathway gets modified so that a new end product is obtained.

b. It produces a protein that is the product of interest.

c. It produces a protein that on its own produce the desired phenotype.

d. It prevents the expression of an already existing gene.

Application - 6. Sustainable Agriculture:
Human population is ever increasing and because of this the major challenge for agricultural scientists is to increase the food production at almost the same pace. This is not possible by the conventional methods used for increasing and improving the yield. In recent years, it has been understood that biotechnology can play a major role to overcome this problem.

Table 4 Transgenic plants that produce antigens.

Transgenic plants	Active against bacterial/viral pathogens	Antigens (vaccines)
Potato	*E.coli*	Heat labile enterotoxin β-subunit
	Vibrio cholerae	Cholera toxin β-subunit
Tobacco	Hepatitis B virus	Hepatitis B surface antigen (HBsAg)
	Vibrio cholerae	Cholera toxin β-subunit
Tomato	Rabies virus	Rabies virus glycoprotein

Earlier emphasis was laid on the use of pesticides and fertilisers. Later it was realised that use of pesticides and fertilisers led to environmental pollution. Due to this reason such practices cannot be continued for indefinite period of time. The only way to overcome this is to switch over to sustainable agriculture.
Sustainable agriculture means to use the resources in judicious manner where we do not exploit it to support the present requirement and take care to leave behind sufficient resources for the coming generation. Care should also be taken to use renewable resources. Development which minimises the use of non-renewable resources results in minimising the environmental exploitation.
Through genetic manipulation crop production and usefulness of the products can be increased. Due to the introduction of agricultural biotechnology we have plants which have not one but many improved traits. The only problem which is faced while commercialising these varieties is that with the passage of time their expression is reduced. To overcome this problem scientists cross different transgenic lines that have improved breeding material. The progeny so formed undergoes selfing to give rise to varieties which have desired characters. By the above

technique we can have traits that are all of same size, colour, weight and shape. Their nutritional value can also be improved keeping intact their time of ripening.

Genetically Modified Organisms:

A genetically modified organism (GMO) is any organism whose genetic material has been altered using genetic engineering techniques (i.e., a genetically engineered organism). GMOs are used to produce many medications and genetically modified foods and are widely used in scientific research and the production of other goods. The term GMO is very close to the technical legal term, 'living modified organism', defined in the Cartagena Protocol on Biosafety, which regulates international trade in living GMOs (specifically, "any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology").A more specifically defined type of GMO is a "transgenic organism." This is an organism whose genetic makeup has been altered by the addition of genetic material from an unrelated organism. This should not be confused with the more general way in which "GMO" is used to classify genetically altered organisms, as typically GMOs are organisms whose genetic makeup has been altered without the addition of genetic material from an unrelated organism. The first genetically modified mouse was created in 1974, and the first plant was produced in 1983.

Applications of GMOs:

GMOs are used in biological and medical research, production of pharmaceutical drugs,[experimental medicine (e.g. gene therapy and vaccines against the Ebola virus), and agriculture (e.g. golden rice, resistance to herbicides), with developing uses in conservation. The term "genetically modified organism" does not always imply, but can include, targeted insertions of genes from one species into another. For example, a gene from a jellyfish, encoding a fluorescent protein called GFP, or green fluorescent protein, can be physically linked and thus co-expressed with mammalian genes to identify the location of the protein encoded by the GFP-tagged gene in the mammalian cell. Such methods are useful tools for biologists in many areas of research, including those who study the mechanisms of human and other diseases or fundamental biological processes in eukaryotic or prokaryotic cells.

Controversy:

There is controversy over GMOs, especially with regard to their use in producing food. The dispute involves buyers, biotechnology companies, governmental regulators, nongovernmental organizations, and scientists. The key areas of controversy related to GMO food are whether GM food should be labelled, the role of government regulators, the effect of GM crops on health and the environment, the effect on pesticide resistance, the impact of GM crops for farmers, and the role of GM crops in feeding the world population. In 2014, sales of products that had been labelled as non-GMO grew 30 percent to $1.1 billion.

There is a scientific consensus that currently available food derived from GM crops poses no greater risk to human health than conventional food, but that each GM food needs to be tested on a case-by-case basis before introduction. Nonetheless, members of the public are much less likely than scientists to perceive GM foods as safe. The legal and regulatory status of GM foods varies by country, with some nations banning or restricting them, and others permitting them with widely differing degrees of regulation.

No reports of ill effects have been proven in the human population from ingesting GM food. Although labelling of GMO products in the marketplace is required in many countries, it is not required in the United States and no distinction between marketed GMO and non-GMO foods is recognized by the US FDA. In a May 2014 article in The Economist it was argued that, while GM foods could potentially help feed 842 million malnourished people globally, laws such as the one passed in Vermont, to require labelling of foods containing genetically modified ingredients, could have the unintended consequence of interrupting the process of spreading GM technologies to impoverished countries that suffer with food security problems.
The Organic Consumers Association, and the Union of Concerned Scientists, and Greenpeace stated that risks have not been adequately identified and managed, and they have questioned the objectivity of regulatory authorities. Some health groups say there are unanswered questions regarding the potential long-term impact on human health from food derived from GMOs, and propose mandatory labelling or a moratorium on such products. Concerns include contamination of the non-genetically modified food supply, effects of GMOs on the environment and nature, the rigor of the regulatory process, and consolidation of control of the food supply in companies that make and sell GMOs, or concerns over the use of herbicides with glyphosate.

Regulation of GMOs:

The regulation of genetic engineering varies widely by country. Countries such as the United States, Canada, Lebanon and Egypt use substantial equivalence as the starting point when assessing safety, while many countries such as those in the European Union, Brazil and China authorize GMO cultivation on a case-by-case basis. Many countries allow the import of GM food with authorization, but either do not allow its cultivation (Russia, Norway, Israel) or have provisions for cultivation, but no GM products are yet produced (Japan, South Korea). Most countries that do not allow for GMO cultivation do permit research.
One of the key issues concerning regulators is whether GM products should be labelled. Labelling of GMO products in the marketplace is required in 64 countries. Labelling can be mandatory up to a threshold GM content level (which varies between countries) or voluntary. A study investigating voluntary labelling in South Africa found that 31% of products labelled as GMO-free had a GM content above 1.0%.In Canada and the USA labelling of GM food is voluntary, while in Europe all food (including processed food) or feed which contains greater than 0.9% of approved GMOs must be labelled. There is a scientific consensus that currently available food derived from GM crops poses no greater risk to human health than conventional food, but that each GM food needs to be tested on a case-by-case basis before introduction. Nonetheless, members of the public are much less likely than scientists to perceive GM foods as safe. The legal and regulatory status of GM foods varies by country, with some nations banning or restricting them, and others permitting them with widely differing degrees of regulation. There is no evidence to support the idea that the consumption of approved GM food has a detrimental effect on human health. Some scientists and advocacy groups, such as Greenpeace and World Wildlife Fund, have however called for additional and more rigorous testing for GM food.

Examples:

a. Microbes:

Bacteria were the first organisms to be modified in the laboratory, due to the relative ease of modifying their genetics. They continue to be important model organisms for experiments in genetic engineering. In the field of synthetic biology, they have been used to test various synthetic approaches, from synthesizing genomes to creating novel nucleotides. These organisms are now used for several purposes, and are particularly important in producing large amounts of pure human proteins for use in medicine. Genetically modified bacteria are used to produce the protein insulin to treat diabetes. Similar bacteria have been used to produce biofuels, clotting factors to treat haemophilia, and human growth hormone to treat various forms of dwarfism.

In 2017 researchers genetically modified a virus to express spinach defensin proteins. The virus was injected into orange trees to combat citrus greening disease that had reduced orange production 70% since 2005. In addition, various genetically engineered micro-organisms are routinely used as sources of enzymes for the manufacture of a variety of processed foods. These include alpha-amylase from bacteria, which converts starch to simple sugars, chymosin from bacteria or fungi, which clots milk protein for cheese making, and pectinesterase from fungi, which improves fruit juice clarity.

b. Plants:

Transgenic plants have been engineered for scientific research, to create new colours in plants, and to create different crops.

In research, plants are engineered to help discover the functions of certain genes. One way to do this is to knock out the gene of interest and see what phenotype develops. Another strategy is to attach the gene to a strong promoter and see what happens when it is over expressed. A common technique used to find out where the gene is expressed is to attach it to GUS or a similar reporter gene that allows visualisation of the location.'

After thirteen years of collaborative research, an Australian company – Florigene, and a Japanese company – Suntory, created a blue rose (actually lavender or mauve) in 2004.The genetic engineering involved three alterations – adding two genes, and interfering with another. One of the added genes was for the blue plant pigment delphinidin cloned from the pansy. The researchers then used RNA interference (RNAi) technology to depress all colour production by endogenous genes by blocking a crucial protein in colour production, called dihydroflavonol 4-reductase (DFR), and adding a variant of that protein that would not be blocked by the RNAi but that would allow the delphinidin to work. The roses are sold in Japan, the United States, and Canada. Florigene has also created and sells lavender-coloured carnations that are genetically engineered in a similar way. Simple plants and plant cells have been genetically engineered for production of biopharmaceuticals in bioreactors as opposed to cultivating plants in open fields. Work has been done with duckweed Lemna minor, the algae *Chlamydomonas reinhardtii* and the moss *Physcomitrella patens*. An Israeli company, Protalix, has developed a method to produce therapeutics in cultured transgenic carrot and tobacco cells. Protalix and its partner, Pfizer, received FDA approval to market its drug Elelyso, a treatment for Gaucher's disease, in 2012.

c. Invertebrates:

Fruit flies

In biological research, transgenic fruit flies (Drosophila melanogaster) are model organisms used to study the effects of genetic changes on development. Fruit flies are often preferred over other animals due to their short life cycle, low maintenance requirements, and relatively simple genome compared to many vertebrates.

Mosquitoes

In 2010, scientists created "malaria-resistant mosquitoes" in the laboratory. The World Health Organization estimated that malaria killed almost one million people in 2008. Genetically modified male mosquitoes containing a lethal gene have been developed to combat the spread of dengue fever and the Zika virus. Aedes aegypti mosquitoes, the single most important carrier of dengue fever and the Zika virus, were reduced by 80% in a 2010 trial of these GM mosquitoes in the Cayman Islands and by 90% in a 2015 trial in Bahia, Brazil. In comparison, the Florida Keys Mosquito Control District has achieved only 30–60% population reduction with traps and pesticide spraying. In 2016 FDA approved a genetically modified mosquito intervention for Key West, Florida. UK firm Oxitec proposed the release of millions of modified male (non-biting) mosquitoes to compete with wild males for mates. The males are engineered so that their offspring die before maturing, helping to eradicate mosquito-borne disease. Final approval was to be based on a local referendum to be held in November. Andrea Crisanti, a molecular biologist at Imperial College in London is working on ways to stop the *A. gambiae* mosquito from transmitting disease.

Bollworms

A strain of *Pectinophora gossypiella* (Pink bollworm) has been genetically engineered to express a red fluorescent protein. This allows researchers to monitor bollworms that have been sterilized by radiation and released to reduce bollworm infestation. The strain has been field tested for over three years and has been approved for release.

Cnidaria

Cnidaria such as Hydra and the sea anemone *Nemato stellavectensis* are attractive model organisms to study the evolution of immunity and certain developmental processes. An important technical breakthrough was the development of procedures for generation of stable transgenic hydras and sea anemones by embryo microinjection.

d. Chordates :

Fishes:

Genetically modified fish are used for scientific research and as pets, and are being considered for use as food and as aquatic pollution sensors.GM fish are widely used in basic research in genetics and development. Two species of fish, zebrafish and medaka, are most commonly modified because they have optically clear chorions (membranes in the egg), rapidly develop, and the 1-cell embryo is easy to see and microinject with transgenic DNA.

The GloFish is a patented brand of genetically modified (GM) fluorescent zebrafish with bright red, green, and orange fluorescent color. Although not originally developed for the

ornamental fish trade, it became the first genetically modified animal to become publicly available as a pet when it was introduced for sale in 2003. They were quickly banned for sale in California.

GM fish have been developed with promoters driving an over-production of "all fish" growth hormone for use in the aquaculture industry to increase the speed of development and potentially reduce fishing pressure on wild stocks. This has resulted in dramatic growth enhancement in several species, including salmon, trout and tilapia. AquaBounty Technologies, a biotechnology company working on bringing a GM salmon to market, claims that their GM AquAdvantage salmon can mature in half the time as wild salmon. AquaBounty applied for regulatory approval to market their GM salmon in the US, and was approved in November 2015. On 25 November 2013 Canada approved commercial scale production and export of GM Salmon eggs but they are not approved for human consumption in Canada. Several academic groups have been developing GM zebrafish to detect aquatic pollution. The lab that originated the GloFish discussed above originally developed them to change colour in the presence of pollutants, to be used as environmental sensors. A lab at University of Cincinnati has been developing GM zebrafish for the same purpose, as has a lab at Tulane University. Recent research on pain in fish has resulted in concerns being raised that genetic-modifications induced for scientific research may have detrimental effects on the welfare of fish.

Amphibians:
Genetically modified frogs are used for scientific research and are widely used in basic research including genetics and early development. Two species of frog, *Xenopus laevis* and *Xenopus tropicalis*, are most commonly used. GM frogs are also being used as pollution sensors, especially for endocrine disrupting chemicals.

Mammals:
Genetically modified mammals are an important category of genetically modified organisms. Ralph L. Brinster and Richard Palmiter developed the techniques responsible for transgenic mice, rats, rabbits, sheep, and pigs in the early 1980s, and established many of the first transgenic models of human disease, including the first carcinoma caused by a transgene. The process of genetically engineering animals is a slow, tedious, and expensive process. However, new technologies are making genetic modifications easier and more precise. The first transgenic (genetically modified) animal was produced by injecting DNA into mouse embryos then implanting the embryos in female mice. Genetically modified animals currently being developed can be placed into six different broad classes based on the intended purpose of the genetic modification:
1. to research human diseases (for example, to develop animal models for these diseases);
2. to produce industrial or consumer products (fibres for multiple uses);
3. to produce products intended for human therapeutic use (pharmaceutical products or tissue for implantation);
4. to enrich or enhance the animals' interactions with humans (hypo-allergenic pets);
5. to enhance production or food quality traits (faster growing fish, pigs that digest food more efficiently);
6. to improve animal health (disease resistance)

Gene Knockouts:

This technique is used to study the function of the gene by making it nonfunctional. The principle behind this is simple. Under normal condition a particular gene is doing its function by the production of RNA from transcription and, finally the protein by translation. By inactivating the gene (gene knockout), we are able to switch-off the gene and the phenotype of the organism can be studied in the absence of the product made from that particular gene.

There are various ways to knockout a gene, by disrupting the gene in the genome, by deleting the whole or part of the gene, or by inserting an additional DNA in the gene, which act as an insulator in the transcription. These methods of Gene knockout are now becoming very powerful tools in the study of the genome and also the function of individual genes.

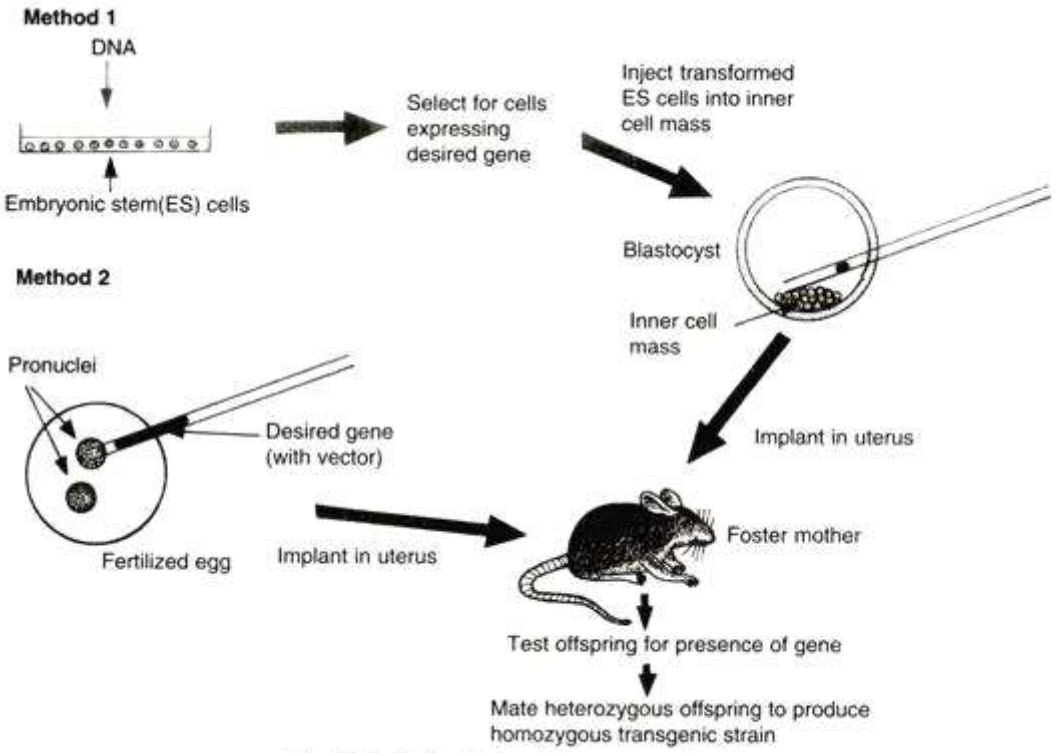


Fig. 18.4. Method of producing transgenic mice.

Mouse disease models:

Because of their phylogenetic relatedness and physiological similarity to humans, the ease of maintaining and breeding them in the laboratory, and the availability of many inbred strains, house mice, Mus musculus, have long served as models of human biology and disease. Genomic studies have highlighted the striking genetic homologies between the two species. These studies, together with the development of methods for the creation of transgenic, knockout, and knock-in mice, have provided added impetus and powerful tools for mouse research, and have led to a dramatic increase in the use of mice as model organisms. Studies on mice have contributed immeasurably to our understanding of human biology. All too often, however, mice respond to experimental interventions in ways that differ markedly from humans. Endostatin, the anticancer drug alluded to in the epigraph, is but one of many treatments that cure cancer in mice but have limited effectiveness in humans. Indeed, the majority of oncology drugs that enter clinical trials never reach the marketplace. There are many reasons for the high failure rate of drug development, but the limitations of the animal

models used in drug testing are an important factor. Many substances that are carcinogens in mice are not carcinogenic in humans—and vice versa. Moreover, mouse strains that were created to mimic human genetic diseases frequently have phenotypes that differ from their human counterparts. Because of the assumption that mice will serve as reliable models for humans, differences between the two species are often greeted with surprise as well as dismay. But these differences should not elicit surprise; indeed, they should be expected. The lineages leading to modern rodents and primates are thought to have diverged from a common ancestral species that lived some 85 million years ago. Since that time, species in these lineages evolved in and became adapted to very different environments.

The use of model organisms in biological research is based on the concept of unity in biology, a concept expressed most famously in Jacques Monod and François Jacob's aphorism, "Anything found to be true of E. coli must also be true of elephants" . But biology is characterized by diversity as well as unity; evolution is "descent with modification" . The art of choosing model organisms involves recognizing the properties of these organisms that they are likely to share with organisms of other species—especially, for biomedical research, humans. Monod and Jacob were concerned with genetic regulatory mechanisms and other basic biological processes that must have arisen very early in the evolutionary history of living organisms and so are similar in bacteria and in mammals. Mice have served and will continue to serve as valuable models for the study of basic biological processes that, in Wimsatt's terms, became developmentally entrenched before the rodent and primate lineages diverged and have been conserved during the separate evolutionary histories of mice and humans.

Studies of the immune system highlight both the value of mouse research in elucidating common features of mammalian biology as well as the limitations of translating this research in areas in which humans are likely to differ from mice. Research on mice has contributed greatly to our knowledge of the adaptive immune system; mouse research has led to the discovery of the major histocompatibility complex genes and the T cell receptor, and to our understanding of the regulation of antibody synthesis and many other features of the immune system. But there are many differences between the mouse and human immune systems, such that much research on immunological diseases in mice is not transferable to humans, and many immunologists are now calling for a return to the study of human immunology. From an evolutionary perspective, this is understandable. The adaptive immune system evolved in jawed fish some hundreds of million years before the evolution of mammals. Many features of this ancestral immune system, including antigen recognition, generation of antibody diversity, clonal selection, and immunological tolerance, are critical for survival and have been maintained in most or all of the descendants of these early vertebrates. On the other hand, species differences in the mechanisms for the maintenance of memory T cells must have evolved in response to the evolution of different life spans. Moreover, specific features of the immune system evolve rapidly, as host species coevolve with their pathogens and commensal microbiota . Since humans and mice harbour different sets of pathogens and microbiomes, it is not surprising that host–pathogen and host–microbiome coevolution has led to differences between the human and mouse immune systems.

The fact that the highly conserved mammalian genome can give rise to a wide variety of different species indicates that the relationships between genotype and phenotype differ among mammalian species. Comparisons between mice and humans are invaluable for understanding the developmental mechanisms that lead to such different genotype–phenotype relationships. Some of the genetic differences between mice and humans are differences in coding sequences,

which give rise to proteins with different properties. For example, mouse haemoglobin has a lower affinity for O2 than does human haemoglobin, which facilitates the dissociation of O2 from haemoglobin in peripheral tissues and helps to support the higher metabolic rate in mice. Perhaps more importantly, however, are differences in the genetic or epigenetic regulation of gene expression in these species. The expression of potassium channel genes in the heart exemplifies these differences. Mice have a heart rate of ∼600 beats/min, while humans have a resting heart rate of ∼70 beats/min. This difference in heart rate entails that the cardiac action potential be much shorter in mice than in humans. Indeed, the repolarization phase of the cardiac action potential, which is due to outward K+ currents, is much shorter in mice. This difference is due to different contributions of various K+ currents, which in turn are presumably due to differences in expression of K+ channel genes in the two species. Evolved differences in the regulation of gene expression are important because they may involve the rewiring of gene (or protein) networks. Gene networks in mice and humans have similar numbers of nodes (genes) but the connectivity of the nodes in these networks, and the relationships between genes and phenotypes, differ between the two species. The different network architectures and different genotype–phenotype relationships between mice and humans mean that the relationships between genotype and disease are also likely to differ in these two species. Perturbations of gene and protein networks by environmental manipulation as well as by mutation are likely to have different effects on diseases as well as on other phenotypes in mice than in humans. In short, mice are problematic models for understanding human disease. There are other good reasons to pursue research on mice. Although house mice are not a major source of human disease, they can transmit lymphocytic choriomeningitis virus and perhaps other pathogens to humans, and other rodent species are important reservoirs for zoonoses. Research on mice may yield information that will help to prevent or ameliorate these diseases. Finally, mice should be studied for their own sake, to understand their biology and to maintain the health of pet mice, laboratory mice, and wild mice.

Unfortunately, despite the many attempts to translate the results of mouse research to humans, we still cannot specify in advance which research in mice is likely to benefit or shed light on human biology and health. For the most part, we have only anecdotal information about studies in mice that translated to humans and those that did not. We need more systematic collection, reporting and analysis of mouse research (and research on other "model organisms") to figure out what works and what does not. Until we have that information, we need to be more critical in pursuing mouse research and in making claims about the applicability of this research to humans.

In addition to problems resulting from the evolved differences between mice and humans, other aspects of mouse research have compromised the value of this research and have further complicated the extrapolation of mouse research to humans. Thus, e.g., laboratory mice are often housed at temperatures below their thermoneutral zone, and as a result are cold-stressed, sleep deprived, and hypertensive. The biology of laboratory mice may also be affected by their housing in same-sex groups and their lack of opportunities for physical exercise. Although mice are often used as models of diseases of aging, for logistical and financial reasons most mouse research is carried out on young animals. And although mouse cells are more sensitive to oxygen damage than are human cells, cell culture studies are often carried out in 20% oxygen, which is non-physiological and is more damaging to mouse cells than to human cells. Finally, there are no agreed upon standards for the design, analysis, or publication of mouse research (or research with other model organisms). The statistical analysis of studies of mice

and other animals is often substandard, and there may be important publication biases because negative results may not get published. All of these problems need to be addressed before studies on mice can be properly interpreted and extrapolated to humans.
Despite all of the documented differences between mice and humans, and despite the history of "errors in translation" in the application of research on mice to humans, reports of research on mice are frequently accompanied by unwarranted and misleading claims, such as "Furthering our understanding of mouse X should provide novel insights into human Y." Such claims raise false hopes and are ultimately self-defeating, in that they waste resources and increase public skepticism concerning the value of biomedical research. Indeed, the problems of translating research on mice and other model organisms to humans have led a number of scientists to question the value of this research . Furthermore, critical discussions of animal experimentation are routinely distorted by "animal rights" activists to support their belief that this experimentation should be stopped. These intrusions, however unwelcome, should not stifle discussion. For reasons mentioned above, research on mice (and other species) is essential and should be supported. This research should, however, be designed and interpreted with appropriate appreciation of the evolved differences as well as the similarities between *M. musculus* and *H. sapiens*.

Gene Silencing :
Gene silencing is the regulation of gene expression in a cell to prevent the expression of a certain gene. Gene silencing can occur during either transcription or translation and is often used in research. In particular, methods used to silence genes are being increasingly used to produce therapeutics to combat cancer and diseases, such as infectious diseases and neurodegenerative disorders.
Gene silencing is often considered the same as gene knockdown. When genes are silenced, their expression is reduced. In contrast, when genes are knocked out, they are completely erased from the organism's genome and, thus, have no expression. Gene silencing is considered a gene knockdown mechanism since the methods used to silence genes, such as RNAi, CRISPR, or siRNA, generally reduce the expression of a gene by at least 70% but do not completely eliminate it. Methods using gene silencing are often considered better than gene knockouts since they allow researchers to study essential genes that are required for the animal models to survive and cannot be removed. In addition, they provide a more complete view on the development of diseases since diseases are generally associated with genes that have a reduced expression.

Transcriptional Gene Silencing (TGS):
Transcriptional gene silencing (TGS) is a gene silencing mechanism in which inactivation of (trans) gene specific RNA synthesis takes place. These are predominantly observed in transgenic plants containing multiple copies of homologous transgene or endogenous gene.
TGS is characterized by the sequence specific DNA methylation at promoter region. TGS is believed to occur through direct DNA interaction between silencer transgene locus and other loci exhibit homologous sequence in their promoter region. In transgenic plants, it has been demonstrated that de-novo methylation of the transgene promoter sequence results in transcriptional inactivation. The pairing of DNA-DNA probably results in methylation and in turn occurs gene silencing. Although methylation at promoter region is significance of TGS, it is however, presumed that methylation do not alone suppress transcription. DNA methylation

probably induces the transcriptional silencing through chromatin components. The link between DNA methylation and transcriptional inactivation is aided by repressive protein MeCP2 which specifically binds to transgene region. Some proteins of the repressive complex initiates de novo DNA methylation. Increase in methylation is followed by acquiring condensed chromatin structure by transcriptionally silent transgene. This type of chromatin remodelling is believed to be responsible for maintenance of repressive status and in the propagation of non-symmetrical methylation pattern in plants.

Post Transcriptional gene silencing:

Production of dsRNA from inverted transgene repeats and single copy transgene triggers PTGS. It was proposed that dsRNA would be act as template for an RNA-directed RNA polymerase. At least four models have been proposed to explain induction of PTGS. First model is known as threshold model explains multicopy insertion or highly expressed single gene in the threshold concentration.

According to this model, plant cells exhibit surveillance system that can detect mRNA expressed above as certain concentration and acts as templates for RDRP. The viral resistant plants contains high number of transgene copies which results in the transcription of high amount of transgene mRNA. This high concentration of mRNA is easily sensed by plant cells and initiate sequence specific process of transgene mRNA degradation. Second model transgenes are inserted as multi copies. Which trigger ectopic pairing and in turn induce methylation. The whole process is culminated in premature termination of transcription.

Production of this type of RNA known as aberrant RNA. The truncated RNA transcript acts as template for RDRP. The third model envisages of inverted repeat transgene integration duplex formed by the mRNA (self-complementation) is template for RDRP (Fig. 20.3). Analysis of transgene organisation reveals that silencing occurs in plants containing inverted-repeats. (IR) of the transgene. The palindromic sequence formed by IR is able to activate production of aberrant RNA (abRNA) from endogenous homologous via ectopic pairing. Recent studies on PTGS of nitrate reductase (NR) genes strongly suggested that the active transcriptional status of NR genes is indispensable for co-suppression of NR (trans) in transgenic tobacco plant.

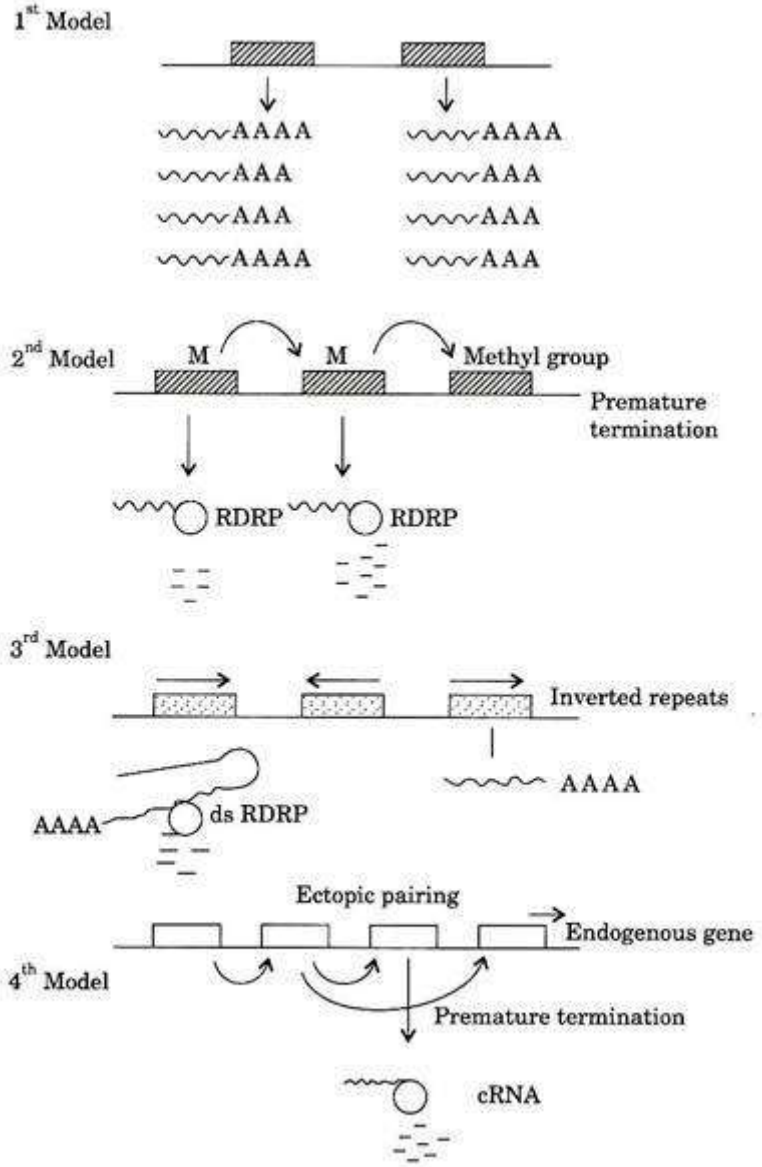


Fig. 20.3 Various models for PTGS

In the last and forth model modification (or methylation) is done to the endogenous gene by ectopic pairing and methylate transgene in inverted repeat and an endogenous gene. Thus modified endogenous gene produce truncated mRNA, which acts as template for RDRP.

Once the transgene mRNA produced. They are potentiating to silence same sense endogenous gene transcript as viral genomic RNA. This is due to plant encoded RNA dependent RNA polymerase which produces small RNA molecules (cRNA) is complementary for transgenic mRNA. The small cRNA potentiate the degradation of the target RNA. Hybridization occurs between their two and target for their degradation mediated by dsRNA or the endonucleases that cleaves ssRNA, which is present adjacent to the dsRNA duplex. The co-suppression takes place by sequence-specific RNA degradation within cytoplasm suggested that PTGS related RNA degradation takes place within cytoplasm.

The possibility of gene silencing by dsRNA has been studied for both virus resistance and co-suppression. In one of the case studies plant expressing transgene sense mRNA derived from virus crossed with plant expressing antisense mRNA of the transgene. Each parent is actually susceptible to virus infection because it contains single hemizygous transgene. All progeny that

inherent both sense and to antisense transgenes are resistant to the virus. The PTGS absolutely requires dsRNA and therefore recognise only mRNA that has larger regions of self-complementary. These are produced by read through transcription of transgene in an inverted repeat configuration. The small cRNA produced by RDRP can easily escape the nucleus to potentiate the degradation of cytoplasmic RNA (Fig. 20.4).

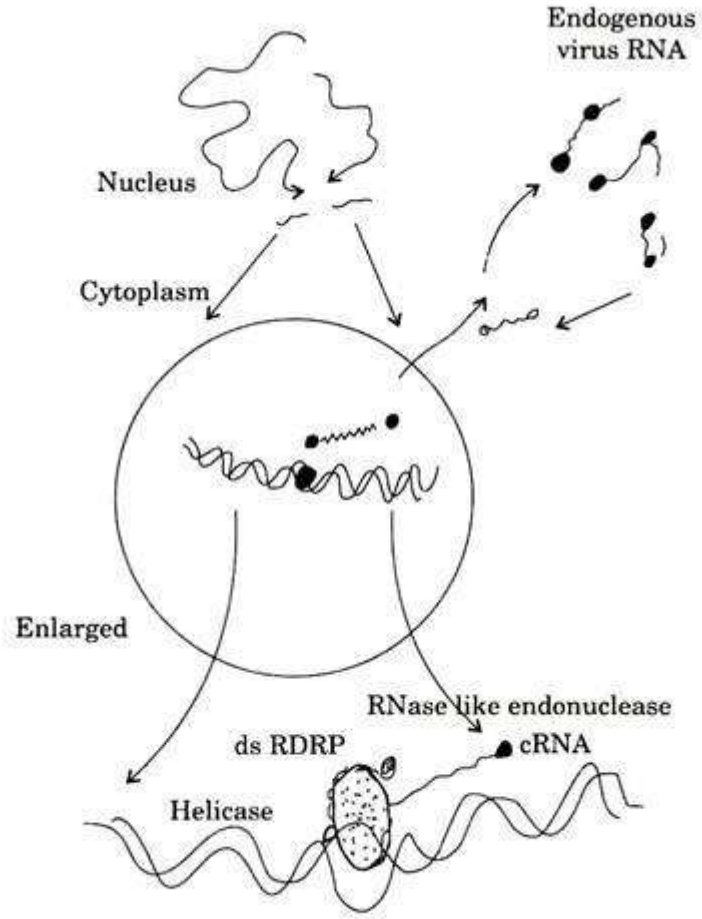


Fig. 20.4 Model for dsRNA induced PTGS (After waterhouse *et al*., 1998, Proc. Natl. Acad. Sci. USA.)

Execution of post-transcriptional gene silencing of endogenous or reporter gene have been described in transgenic plants containing sense or antisense transgenes. In these transgenic plants, either co-suppression or antisense appears to be induction of surveillance system within plant that specifically degrades both transgene and target RNA.

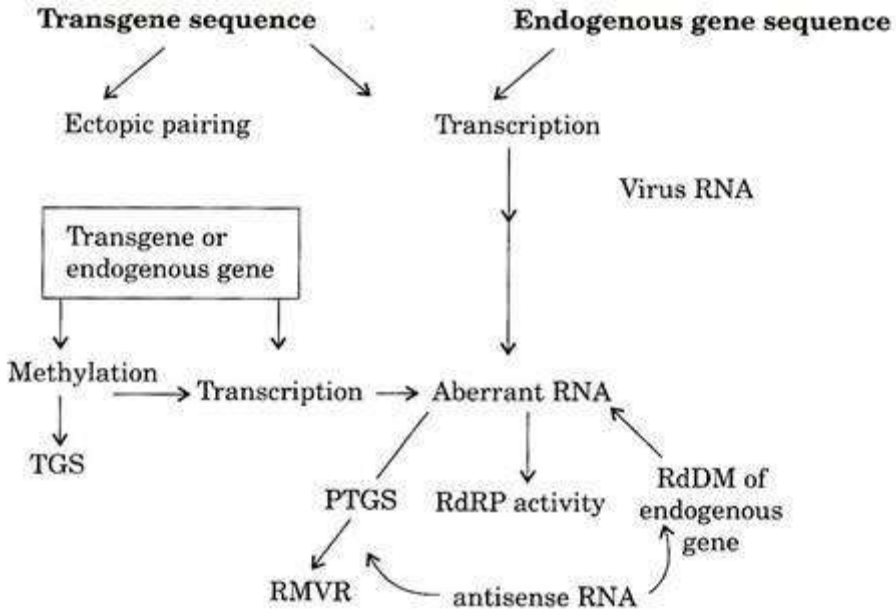


Fig. 20.5 Gene Silencing mechanism of TGS and PTGS

Table 20.1 Relationship between various types of homology-dependent gene silencing and host defence response to invasive sequence (After Kooter *et al.*, 1999)

Types of HDGS	Cell compartment	Invasive sequence	Molecular effect	Possible triggers
TGS	Nucleus	Transposable elements (TE)	DNA hyper methylation	DNA-DNA pairing : IRS
		Retroelement	DNA hyper methylation	(Abb) RNA-DNA
		viroids	DNA hyper methylation	Sequence incompatibility
		bacterial DNA		
		viruses		
		RNA genome or		
PTGS	Cytoplasm	RNA replication intermediate of DNA genome	RNA turn over	(Ab) RNA or over-express RNA

Gene Therapy:

There are two types of gene therapies:

I. Ex vivo gene therapy:

This involves the transfer of genes in cultured cells (e.g., bone marrow cells) which are then reintroduced into the patient.

II. In vivo gene therapy:

The direct delivery of genes into the cells of a particular tissue is referred to as in vivo gene therapy.

Type - I. Ex Vivo Gene Therapy:

The ex vivo gene therapy can be applied to only selected tissues (e.g., bone marrow) whose cells can be cultured in the laboratory. The technique of ex vivo gene therapy involves the following steps (Fig. below).

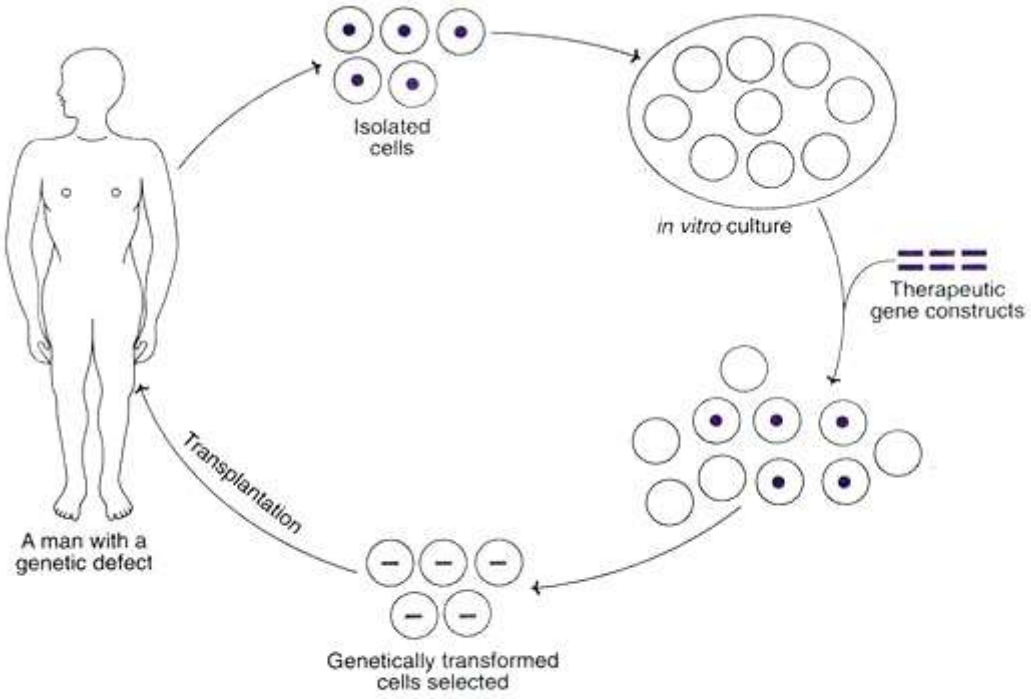


Fig. 13.2 : The procedure for ex vivo gene therapy.

1. Isolate cells with genetic defect from a patient.

2. Grow the cells in culture.

3. Introduce the therapeutic gene to correct gene defect.

4. Select the genetically corrected cells (stable trans-formants) and grow.

5. Transplant the modified cells to the patient.

The procedure basically involves the use of the patient's own cells for culture and genetic correction, and then their return back to the patient. This technique is therefore, not associated with adverse immunological responses after transplanting the cells. Ex vivo gene therapy is efficient only, if the therapeutic gene (remedial gene) is stably incorporated and continuously expressed. This can be achieved by use of vectors.

Vectors in Gene Therapy:

The carrier particles or molecules used to deliver genes to somatic cells are referred to as vectors. The important vectors employed in ex vivo gene therapy are listed below and briefly described next.

i. Viruses
ii. Human artificial chromosome
iii. Bone marrow cells.

i. Viruses:

The vectors frequently used in gene therapy are viruses, particularly retroviruses. RNA is the genetic material in retroviruses. As the retrovirus enters the host cell, it synthesizes DNA from RNA (by reverse transcription). The so formed viral DNA (referred to as provirus) gets incorporated into the DNA of the host cell.

The proviruses are normally harmless. However, there is a tremendous risk, since some of the retroviruses can convert normal cells into cancerous ones. Therefore, it is absolutely essential to ensure that such a thing does not happen.

Making retroviruses harmless:

Researchers employ certain biochemical methods to convert harmful retroviruses to harmless ones, before using them as vectors. For instance, by artificially removing a gene that encodes for the viral envelope, the retrovirus can be crippled and made harmless. This is because, without the envelope, retrovirus cannot enter the host cell. The production of a large number (billions) of viral particles can be achieved, starting from a single envelope defective retrovirus .

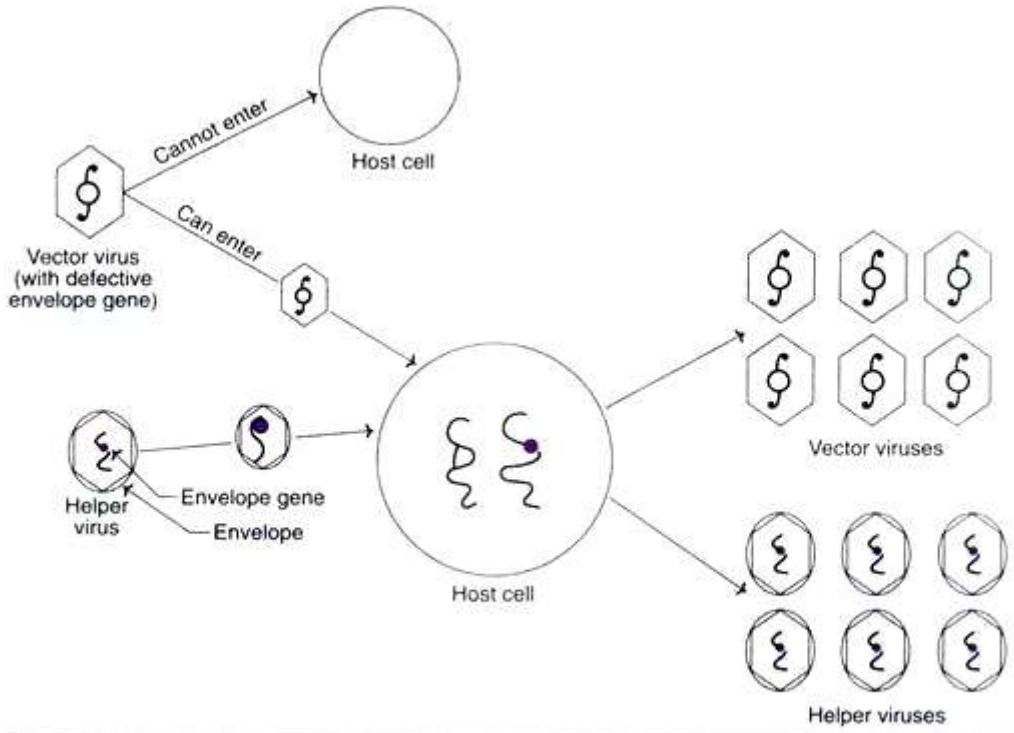


Fig. 13.3 : Large scale production of vector viruses by using helper viruses.

This is made possible by using helper viruses which contain normal gene for envelope formation. Along with the helper virus, the vector (with defective envelope gene) can enter the host cell and both of them multiply. By repeated multiplication in host cells, billions of vector and helper viruses are produced.

The vector viruses can be separated from the helper viruses and purified. Isolation of vector viruses, totally free from helper viruses, is absolutely essential. Contamination of helper viruses is a big threat to the health of the patients undergoing gene therapy.

Retroviruses in gene therapy:

The genetic map of a typical retrovirus is depicted in Fig. 13.4A. In general, the retrovirus particle has RNA as a genome organized into six regions. It has a 5′-long terminal repeat (5′-LTR), a non-coding sequence required for packaging RNA designated as psi (Ψ), a gene gag coding for structural protein, a gene pol that codes for reverse transcriptase, a gene env coding for envelope protein and a 3-LTR sequence.

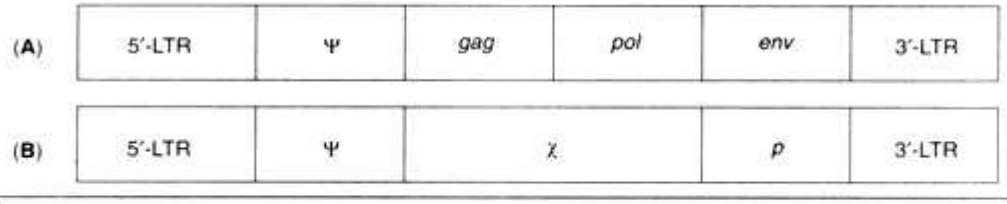


Fig. 13.4 : A retrovirus used in gene therapy. (A) General map of a typical retrovirus (B) Gene map of a modified retrovirus for use in gene therapy (LTR-Long terminal repeat; Ψ-Packaging signal sequence; gag-Coding sequence for structural protein; pol-Coding sequence for reverse transcriptase; env-Envelope protein coding sequence; χ-Therapeutic gene; p–Promoter gene).

For use of a retrovirus as a vector, the structural genes gag and pol are deleted. These genes are actually adjacent to Ψ region. In addition, a promoter gene is also included (Fig. 13.4B). This vector design allows the synthesis of cloned genes. A retroviral vector can carry a therapeutic DNA of maximum size of 8 kb.

A retroviral vector DNA can be used to transform the cells. However, the efficiency of delivery and integration of therapeutic DNA are very low. In recent years, techniques have been developed to deliver the vector RNA to host cells at a high frequency. For this purposes, packaged retroviral RNA particles are used. This technique allows a high efficiency of integration of pharmaceutical DNA into host genome.

Several modified viral vectors have been developed in recent years for gene therapy. These include oncoretrovirus, adenovirus, adeno-associated virus, herpes virus and a number of hybrid vectors combining the good characters of the parental vectors.

Murine leukaemia viruses in gene therapy:

This is a retrovirus that causes a type of leukaemia in mice. It can react with human cells as well as the mouse cells, due to a similarity in the surface receptor protein. Murine leukaemia virus (MLV) is frequently used in gene transfer.

AIDS virus in gene therapy

It is suggested that the human immunodeficiency virus (HIV) can be used as a vector in gene transfer. But this is bound to create public uproar. Some workers have been successful in creating a harmless HIV (crippled HIV) by removing all the genes related to reproduction. At the same time, the essential genes required for gene transfer are retained. There is a distinct advantage with HIV when compared with MLV. MLV is capable of bringing out gene transfer only in dividing cells. HIV can infect even non-dividing cells (e.g., brain cells) and do the job of gene transfer effectively. However, it is doubtful whether HIV can ever be used as a vector.

ii. Human Artificial Chromosome:

The details of human artificial chromosome (HAC) are described elsewhere .HAC is a synthetic chromosome that can replicate with other chromosomes, besides encoding a human protein. As already discussed above, use of retroviruses as vectors in gene therapy is associated with a heavy risk. This problem can be overcome if HAC is used. Some success has been achieved in this direction.

iii. Bone Marrow Cells:

Bone marrow contains totipotent embryonic stem (ES) cells. These cells are capable of dividing and differentiating into various cell types (e.g., red blood cells, platelets, macrophages, osteoclasts, B- and T-lymphocytes). For this reason, bone marrow transplantation is the most widely used technique for several genetic diseases.

And there is every reason to believe that the genetic disorders that respond to bone marrow transplantation are likely to respond to ex vivo gene therapy also. For instance, if there is a gene mutation that interferes with the function of erythrocytes (e.g., sickle-cell anaemia), bone marrow transplantation is done. Bone marrow cells are the potential candidates for gene therapy of sickle-cell anaemia. However, this is not as simple as theoretically stated.

Selected Examples of Ex Vivo Gene Therapy:

Therapy for Adenosine Deaminase Deficiency:

The first and the most publicized human gene therapy was carried out to correct the deficiency of the enzyme adenosine deaminase (ADA). This was done on September 14, 1990 by a team of workers led by Blaese and Anderson at the National Institute of Health, USA (The girl's name is Ashanti, 4 years old then).

Severe combined immunodeficiency (SCID):

This is rare inherited immune disorder associated with T-lymphocytes, and (to a lesser extent) B-lymphocytes dysfunction. About 50% of SCID patients have a defect in the gene (located on chromosome 20, and has 32,000 base pairs and 12 exons) that encodes for adenosine deaminase. In the deficiency of ADA, deoxyadenosine and its metabolites (primarily deoxyadenosine 5′-triphosphate) accumulate and destroy T-lymphocytes.

T-Lymphocytes are essential for body's immunity. Besides participating directly in body's defence, they promote the function of B-lymphocytes to produce antibodies. Thus, the patients of SCID (lacking ADA) suffer from infectious diseases and die at an young age. Previously, the children suffering from SCID were treated with conjugated bovine ADA, or by bone marrow transplantation.

Technique of therapy for ADA deficiency:

The general scheme of gene therapy adopted for introducing a defective gene in the patient has been depicted in Fig 13.2. The same procedure with suitable modifications can also be applied for other gene therapies.

A plasmid vector bearing a pro-viral DNA is selected. A part of the pro-viral DNA is replaced by the ADA gene and a gene (G 418) coding for antibiotic resistance, and then cloned. The

antibiotic resistance gene will help to select the desired clones with ADA gene. A diagrammatic representation of the treatment of ADP deficient patient is depicted in Fig. below.

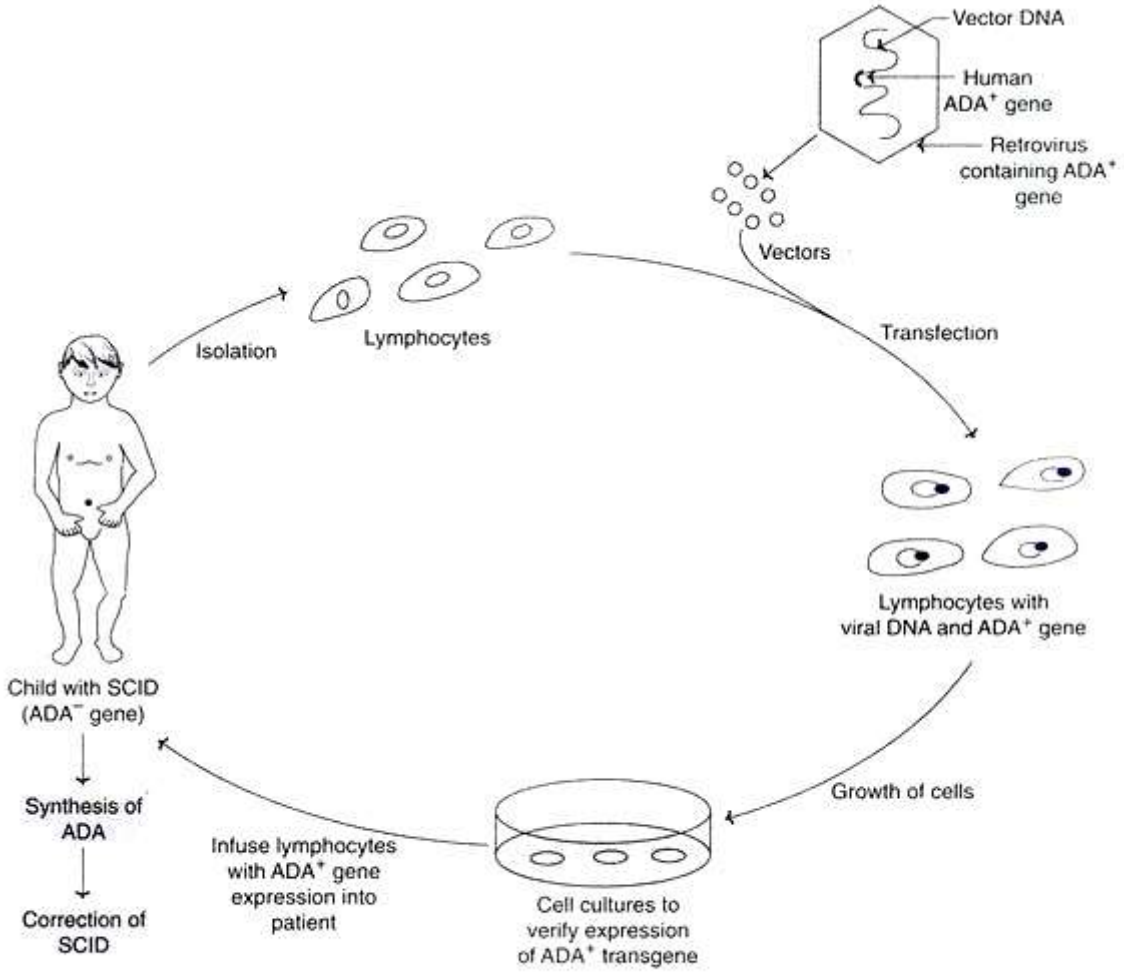


***Fig. 13.5** : Treatment of adenosine deaminase (ADA) deficient patient by somatic* ex vivo *gene therapy (SCID-Severe combined immunodeficiency disease).*

Circulating lymphocytes are removed from a patient suffering from ADA deficiency. These cells are transfected with ADA gene by exposing to billions of retroviruses carrying the said gene. The genetically-modified lymphocytes are grown in cultures to confirm the expression of ADA gene and returned to the patient. These lymphocytes persist in the circulation and synthesize ADA.

Consequently, the ability of the patient to produce antibodies is increased. However, there is a limitation. The lymphocytes have a short life span (just live for a few months), hence the transfusions have to be carried out frequently.

Transfer of ADA gene into stem cells:
In 1995, ADA gene was transferred into the stem cells, obtained from the umbilical cord blood, at the time of baby's delivery. Four days after birth, the infant received the modified cells back. By this way, a permanent population of ADA gene producing cells was established.

Therapy for Familial Hypercholesterolemia:
The patients of familial hypercholesterolemia lack the low density lipoprotein (LDL) receptors on their liver cells. As a result, LDL cholesterol is not metabolised in liver. The accumulated LDL- cholesterol builds up in the circulation, leading to arterial blockage and heart diseases.
Attempts are being made by gene therapists to help the victims of familial hypercholesterolemia. In fact, there is some success also. In a woman, 15% of the liver was removed. The hepatocytes were transduced with retroviruses carrying genes for LDL receptors. These genetically modified hepatocytes were infused into the patient's liver.
The hepatocytes established themselves in the liver and produced functional LDL-receptors. A significant improvement in the patient's condition, as assessed by estimating the lipid parameters in blood, was observed. Further, there were no antibodies produced against the LDL-receptor molecules, clearly showing that the genetically modified liver cells were accepted.

Therapy for Lesch-Nyhan Syndrome:
Lesch-Nyhan syndrome is an inborn error in purine metabolism due to a defect in a gene that encodes for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HCPRT). In the absence of HGPRT, purine metabolism is disturbed and uric acid level builds up, resulting in severe gout and kidney damage. The victims of Lesch- Nyhan syndrome exhibit symptoms of mental retardation, besides an urge to bite lips and fingers, causing self-mutilation.
By using retroviral vector system, HGPRT producing genes were successfully inserted into cultured human bone marrow cells. The major problem in humans is the involvement of brain. Experiments conducted in animals are encouraging. However, it is doubtful whether good success can be achieved by gene therapy for Lesch-Nyhan syndrome in humans, in the near future.

Therapy for Haemophilia:
Haemophilia is a genetic disease due lack of a gene that encodes for clotting factor IX. It is characterized by excessive bleeding. By using a retroviral vector system, genes for the synthesis of factor IX were inserted into the liver cells of dogs. These dogs no longer displayed the symptoms of haemophilia.

Ex Vivo Gene Therapy with Non-Autologous Cells:
The ex vivo gene therapies described above are based on the transplantation of genetically modified cells for the production of desired proteins. However, there are several limitations in using the patient's own cells (autologous cells) for gene therapy. These include lack of enough cells from target tissues, defective uptake of genes and their inadequate expression. To overcome these problems, attempts are on to develop methods to use non-autologous cells (i.e., cells from other individuals or animals). The outline of the procedure is briefly described below.

Tissue-specific cells capable of growing in culture are selected. These include fibroblasts from skin, hepatocytes from liver, and myoblasts from muscle and astrocytes from brain. These cells are cultured and genetically modified with the therapeutic gene. They are then encapsulated in artificial membrane composed of a synthetic polymer (e.g., polyether sultone, alginase-poly L-lysine-alginate). The polymeric membranes are non-immunogenic, therefore the patient can accept non-autologous encapsulated cells. Further, being semipermeable in nature, these membranes allow the nutrients to enter in, and the encoded protein (by the therapeutic gene) to pass out. Experiments conducted in animals have shown some encouraging results for using non-autologous cells in gene therapy. The encapsulated cells were found to proliferate and produce the required protein. However, the success has been very limited in human trials.

Type - II. In Vivo Gene Therapy:

The direct delivery of the therapeutic gene (DNA) into the target cells of a particular tissue of a patient constitutes in vivo gene therapy (see Fig. below). Many tissues are the potential candidates for this approach. These include liver, muscle, skin, spleen, lung, brain and blood cells. Gene delivery can be carried out by viral or non- viral vector systems. The success of in vivo gene therapy mostly depends on the following parameters

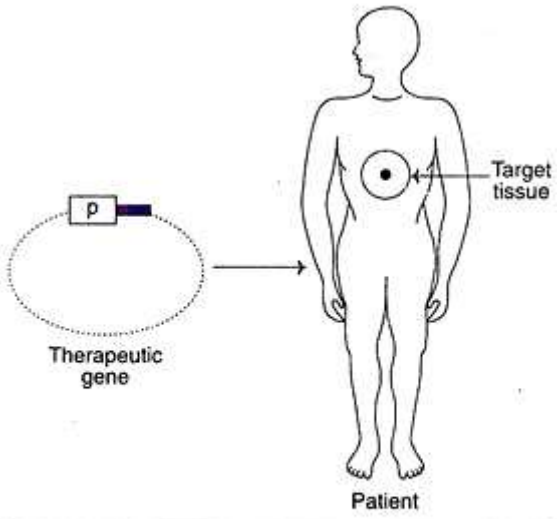


Fig. 13.6 : Diagrammatic representation of in vivo gene therapy. (p-Promoter gene specific for therapeutic gene)

i. The efficiency of the uptake of the remedial (therapeutic) gene by the target cells.
ii. Intracellular degradation of the gene and its uptake by nucleus.
iii. The expression capability of the gene.
In vivo gene therapy with special reference to gene delivery systems (viral, non-viral) with suitable examples is described.

Gene Delivery by Viruses:
Many viral vector systems have been developed for gene delivery. These include retroviruses, adenoviruses, adeno-associated viruses and herpes simplex virus.

Retrovirus vector system:

Replication defective retrovirus vectors that are harmless are being used. A plasmid in association with a retrovirus, a therapeutic gene and a promoter is referred to as plasmovirus. The plasmovirus is capable of carrying a DNA (therapeutic gene) of size less than 3.4 kb. Replication defective virus particles can be produced from the plasmovirus.

As such, for the delivery of genes by retroviral vectors, the target cells must be in a dividing stage. But majority of the body cells are quiescent. In recent years, viral vectors have been engineered to infect non-dividing cells. Further, attempts are on to include a DNA in the retroviral vectors (by engineering env gene) that encodes for cell receptor protein. If this is successfully achieved, the retroviral vector will specifically infect the target tissues.

Adenoviral vector system:

Adenoviruses (with a DNA genome) are considered to be good vectors for gene delivery because they can infect most of the non-dividing human cells. A common cold adenovirus is a frequently used vector. As the target cells are infected with a recombinant adenovirus, the therapeutic gene (DNA) enters the nucleus and expresses itself.

However, this DNA does not integrate into the host genome. Consequently, adenoviral based gene therapy required periodic administration of recombinant viruses. The efficiency of gene delivery by adenoviruses can be enhanced by developing a virus that can specifically infect target cells. This is possible by incorporating a DNA encoding a cell receptor protein.

Adeno-associated virus vector system:

Adeno-associated virus is a human virus that can integrate into chromosome 19. It is a single-stranded, non-pathogenic small DNA virus (4.7 kb). As the adeno-associated virus enters the host cell, the DNA becomes double- stranded, gets integrated into chromosome and expresses. Adeno-associated viruses can serve as good vectors for the delivery of therapeutic genes. Recombinant viruses are created by using two plasmids and an adenovirus (i.e., helper virus) by a special technique. Some attempts were made to use therapeutic genes for the treatment of the human diseases-hemophilia (for production of blood clotting factor IX) and cystic fibrosis (for synthesis of cystic fibrosis trans membrane regulator protein) by employing adeno-associated viruses.

Therapy for cystic fibrosis:

Cystic fibrosis (CF) is one of the most common (frequency 1: 2,500) and fatal genetic diseases. It is characterized by the accumulation of sticky, dehydrated mucus in the respiratory tract and lungs. Patients of CF are highly susceptible to bacterial infections in their lungs and most of them die before reaching the age of thirty.

In the normal persons the chloride ions of the cells are pushed out through the participation of a protein called cystic fibrosis trans membrane regulator (CFTR). In the patients of cystic fibrosis, the CFTR protein is not produced due to a gene defect. Consequently, the chloride ions concentrate within the cells which draw water from the surroundings. As a result, the respiratory tract and the lungs become dehydrated with sicky mucus, an ideal environment for bacterial infections.

As the defective gene for cystic fibrosis was identified in 1989, researchers immediately started working on gene therapy for this disease. Adenoviral vector systems have been used, although the success has been limited. The major drawback is that the benefits are short-lived, since the

adenoviruses do not integrate themselves into host cells. Multiple administration of recombinant adenovirus caused immunological responses that destroyed the cells.
By using adeno-associated virus vector system, some encouraging results were reported in the gene therapy of CF. In the phase I clinical trials with CF patients, the vector persisted for about 70 days and some improvement was observed in the patients. Some researchers are trying to insert CF gene into the developing fetal cells (in experimental animals such as mice) to produce CFTR protein. But a major breakthrough is yet to come.

Herpes simplex virus vector system:
The retroviruses and adenoviruses employed in in vivo gene therapy are engineered to infect specific target cells. There are some viruses which have a natural tendency to infect a particular type of cells. The best example is herpes simplex virus (HSV) type I, which infects and persists in non-dividing nerve cells. HSV is a human pathogen that causes (though rarely) cold sores and encephalitis.
These are a large number of diseases (metabolic, neurodegenerative, immunological, tumors) associated with nervous system. HSV is considered as an ideal vector for in vivo gene therapy of many nervous disorders. The HSV has a double-stranded DNA of about 152 kb length as its genome. About 30 kb of HSV genome can be replaced by a cloned DNA without loss of its basic characteristics (replication, infection, packaging etc.). But there are some technical difficulties in dealing with large-sized DNAs in genetic engineering experiments. Some modified HSV vectors with reduced genomic sizes have been developed.
Most of the work on the gene therapy, related to the use of HSV as a vector, is being conducted in experimental animals. And the results are quite encouraging. HSV vectors could deliver therapeutic genes to the brain and other parts of nervous system. These genes are well expressed and maintained for long periods. More research, however, is needed before going for human trials. If successful, HSV may help to treat many neurodegenerative syndromes such as Parkinson's disease and Alzheimer's disease by gene therapy.

Gene Delivery by Non-Viral Systems:
There are certain limitations in using viral vectors in gene therapy. In addition to the prohibitive cost of maintaining the viruses, the viral proteins often induce inflammatory responses in the host. Therefore, there is a continuous search by researchers to find alternatives to viral vector systems.

Pure DNA constructs:
The direct introduction of pure DNA constructs into the target tissue is quite simple. However, the efficiency of DNA uptake by the cells and its expression are rather low. Consequently, large quantities of DNA have to be injected periodically. The therapeutic genes produce the proteins in the target cells which enter the circulation and often get degraded.

Lipoplexes:
The lipid-DNA complexes are referred to as lipoplexes or more commonly liposomes. They have a DNA construct surrounded by artificial lipid layers. A large number of lipoplexes have been prepared and used. They are non-toxic and non-immunogenic.
The major limitation with the use of lipoplexes is that as the DNA is taken up by the cells, most of it gets degraded by the lysosomes. Thus, the efficiency of gene delivery by lipoplex is very

low. Some clinical trials using liposome-CFTR gene complex showed that the gene expression was very short-lived.

DNA-molecular conjugates:

The use of DNA-molecular conjugates avoids the lysosomal breakdown of DNA. Another advantage of using conjugates is that large-sized therapeutic DNAs (> 10 kb) can be delivered to the target tissues. The most commonly used synthetic conjugate is poly-L-lysine, bound to a specific target cell receptor. The therapeutic DNA is then made to combine with the conjugate to form a complex .

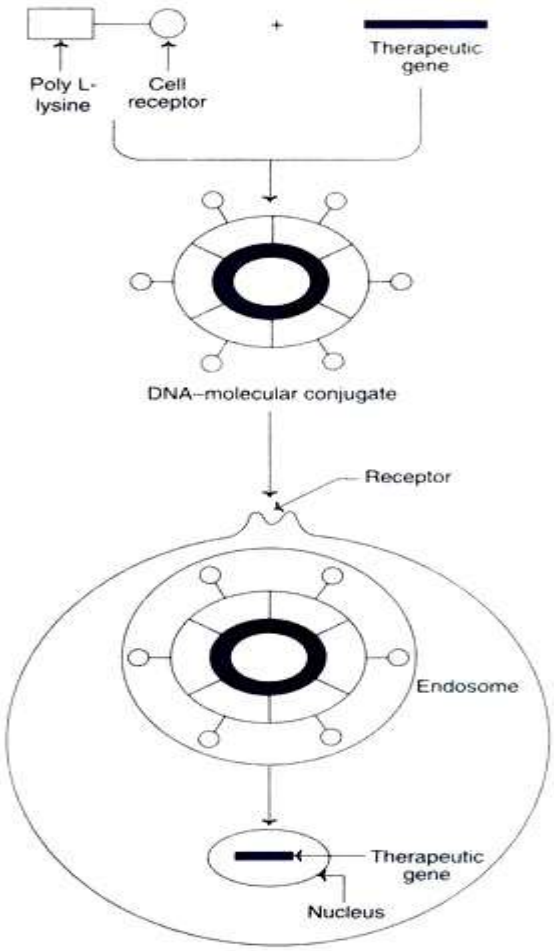


Fig. 13.7 : DNA-molecular conjugate in the delivery of therapeutic gene.

This DNA molecular conjugate binds to specific cell receptor on the target cells. It is engulfed by the cell membrane to form an endosome which protects the DNA from being degraded. The DNA released from the endosome enters the nucleus where the therapeutic gene is expressed.

Human artificial chromosome:

Human artificial chromosome (HAC) which can carry a large DNA one or more therapeutic genes with regulatory elements is a good and ideal vector. Studies conducted in cell cultures using HAC are encouraging. But the major problem is the delivery of the large-sized chromosome into the target cells. Researchers are working to produce cells containing

genetically engineered HAC. There exists a possibility of encapsulating and implanting these cells in the target tissue.

Efficiency of gene delivery by non-viral vectors:
Although the efforts are continuously on to find suitable non-viral vectors for gene delivery, the success has been very limited. This is mainly due to the following two reasons.

1. The efficiency of transfection is very low.
2. The expression of the therapeutic gene is for a very short period, consequently there is no effective treatment of the disease.

Gene Therapy Strategies for Cancer:
Cancer is the leading cause of death throughout the world, despite the intensive treatment strategies (surgery, chemotherapy, radiation therapy). Gene therapy is the latest and a new approach for cancer treatment. Some of the developments are briefly described hereunder.

Tumour necrosis factor gene therapy:
Tumour necrosis factor (TNF) is a protein produced by human macrophages. TNF provides defence against cancer cells. This is brought out by enhancing the cancer-fighting ability of tumour- infiltrating lymphocytes (TILs), a special type of immune cells.
The tumour-infiltrating lymphocytes were transformed with a TNF gene (along with a neomycin resistant gene) and used for the treatment of malignant melanoma (a cancer of melanin producing cells usually occurs in skin). TNF as such is highly toxic, and fortunately no toxic side effects were detected in the melanoma patients injected with genetically altered TILs with TNF gene. Some improvement in the cancer patients was observed.

Suicide gene therapy:
The gene encoding the enzyme thymidine kinase is often referred to as suicide gene, and is used for the treatment of certain cancers. Thymidine kinase (TK) phosphorylates nucleosides to form nucleotides which are used for the synthesis of DNA during cell division. The drug ganciclovir (GCV) bears a close structural resemblance to certain nucleosides (thymidine). By mistake, TK phosphorylates ganciclovir to form triphosphate-GCV, a false and unsuitable nucleotide for DNA synthesis. Triphosphate-GCV inhibits DMA polymerase (Fig. 13.8).

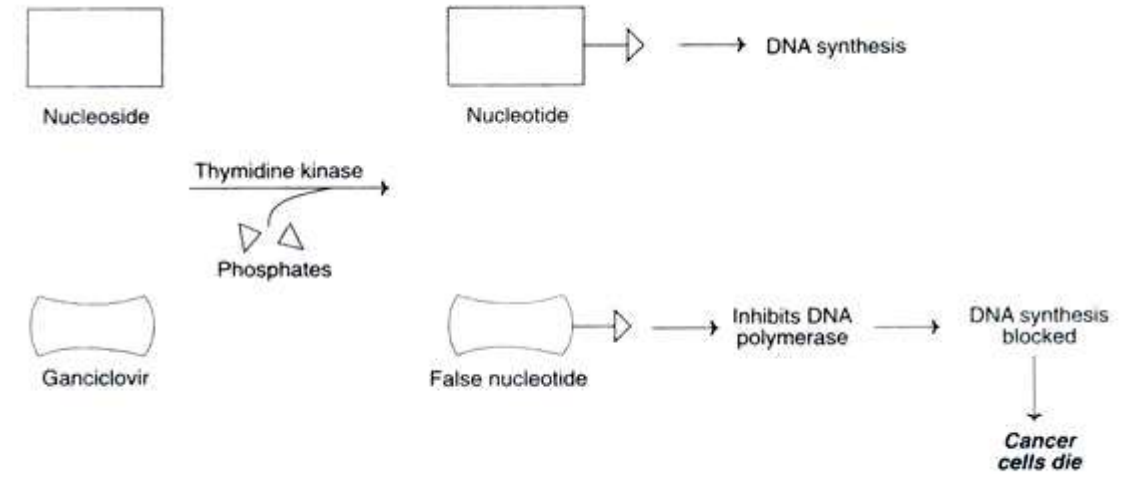


Fig. 13.8 : The action of ganciclovir mediated by thymidine kinase to inhibit the growth of cancer cells.

The result is that the elongation of the DNA molecule abruptly stops at a point containing the false nucleotide (of ganciclovir). Further, the triphosphate-GCV can enter and kill the neighbouring cancer cells, a phenomenon referred to as bystander effect. The ultimate result is that the cancer cells cannot multiply, and therefore die. Thus, the drug ganciclovir can be used to kill the cancer cells.

Ganciclovir is frequently referred to as a pro-drug and this type of approach is called pro-drug activation gene therapy. Ganciclovir has been used for treatment of brain tumours (e.g., glioblastoma, a cancer of glial cells in brain), although with a limited success.

In the suicide gene therapy, the vector used is herpes simplex virus (HSV) with a gene for thymidine kinase (TK) inserted in its genome. Normal brain cells do not divide while the brain tumour cells go on dividing unchecked. Thus, there is a continuous DNA replication in tumour cells. By using GCV-HSVTK suicide gene therapy, some reduction in proliferating tumour cells was reported. Several new strategies are being developed to increase the delivery of HSVTK gene to all the cells throughout a tumour.

Two-gene cancer therapy:

For treatment of certain cancers, two gene systems are put together and used. For instance, TK suicide gene (i.e., GCV-HSVTK) is clubbed with interleukin-2 gene (i.e. a gene promoting immunotherapy). Interleukin-2 produced mobilizes immune response. It is believed that certain proteins are released from the tumour cells on their death.

These proteins, in association with immune cells, reach the tumour and initiate immunological reactions directed against the cancer cells. Two-gene therapies have been carried out in experimental animals with colon cancer and liver cancer, and the results are encouraging.

Gene replacement therapy:

A gene named p^{53} codes for a protein with a molecular weight of 53 kilo Daltons (hence p^{53}). p^{53} is considered to be a tumour-suppressor gene, since the protein it encodes binds with DNA and inhibits replication. The tumour cells of several tissues (breast, brain, lung, skin, bladder, colon, bone) were found to have altered genes of p^{53}(mutated p^{53}), synthesizing different proteins from the original.

These altered proteins cannot inhibit DNA replication. It is believed that the damaged p^{53} gene may be a causative factor in tumour development. Some workers have tried to replace the damaged p^{53}gene by a normal gene by employing adenovirus vector systems .There are some encouraging results in the patients with liver cancer.

Probable Questions:

1. Describe two methods which are used in gene transfer in animal cells?
2. What is lipofection? Describe the procedure?
3. What is gene gun? How biolistic method is used for gene transfer?
4. What is sonoporation?
5. What is optical transfection?
6. What is magnetofection?
7. Write down different applications in Recombinant DNA Technology?
8. What are the applications of GMO ?
9. What is knock out mice? What are its importance in genetic engineering?
10. Explain transcriptional gene silencing?
11. What is post transcriptional gene silencing?
12. What is ex vivo gene therapy? Explain.
13. What is in vivo gene therapy? Explain.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.
8. Brown TA. (2010) Gene Cloning and DNA Analysis. 6th edition. Blackwell Publishing, Oxford, U.K.
9. Primrose SB and Twyman RM. (2006) Principles of Gene Manipulation and Genomics, 7th edition. Blackwell Publishing, Oxford, U.K.
10. Sambrook J and Russell D. (2001) Molecular Cloning-A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Press.

HARD CORE THEORY PAPER (ZHT -412)

MOLECULAR BIOLOGY AND BIOTECHNOLOGY AND TOOLS AND TECHNIQUE

Group B: Tools and Technique used in Molecular Biology

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZHT - 412 (MOLECULAR BIOLOGY AND BIOTECHNOLOGY AND TOOLS AND TECHNIQUE)	VII	**Techniques for Cell Study:** **a. Fluorescent Microscopes, Confocal microscopy, Phase Contrast Microscopy, Dark- Field Microscopy.** **b. Electronic Imaging Systems- Electron Microscopy, TEM Vs. SEM.** **c. Basic concept of flow cytometry.**	1.5	1	1	
	VIII	**Cell fractionation methods:** **a) Preparative Ultracentrifugation** **b) Gradient Centrifugation**		1	1	
	IX	**Separation of Cell Constituents:** **a) Chromatography: ion exchange; gel filtration and HPLC** **b). Electrophoresis-PAGE, SDS-PAGE (One and Two dimensional).**		1	1	
	X	**Spectroscopy:** **Spectrophotometer.** **Pesticide formulation**		1	1	

	XI	**Blotting Methods: Southern, Northern & Western blotting.**		**1**	**1**	
	XII	**Database search tool; Sequence alignment and database searching; Computational tools and biological databases, NCBI, EBL, Sequence similarity tools; Blast and FASTA**		**1**	**1**	

Unit-VII

Techniques for Cell Study: a. Fluorescent Microscopes, Confocal Microscopy, Phase Contrast Microscopy, Dark- Field Microscopy.

b. Electronic Imaging Systems- Electron Microscopy, TEM Vs. SEM.

c. Basic concept of flow cytometry.

Objective: In this unit you will learn about different types of microscope such as Fluorescent microscope, confocal microscope, phase contrast microscope and dark field microscope. Besides optical imaging system you will also learn electronic imaging system, such as SEM ad TEM. Basic concept on flow cytometry will also be discussed here.

A. Fluorescent Microscopes:

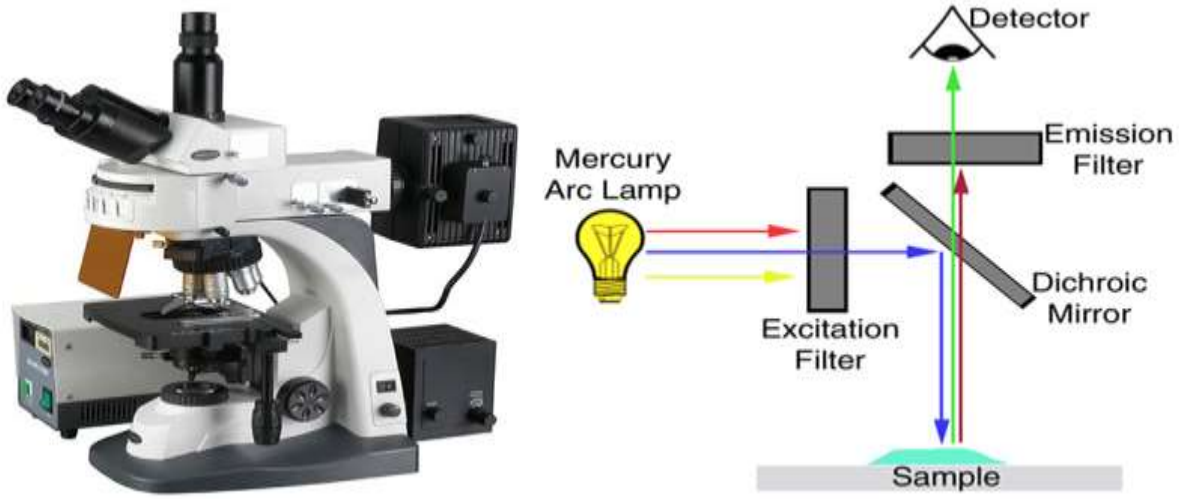
A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence. Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs. The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert, and Heinrich Lehmann, among others.

Principle of fluorescence microscope :

Most cellular components are colourless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with dyes. Fluorescent dyes, also known as fluorophores or fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds.

The emission light can then be filtered from the excitation light to reveal the location of the fluorophores. Fluorescence microscopy uses a much higher intensity light to illuminate the sample. This light excites fluorescence species in the sample, which then emit light of a longer wavelength. The image produced is based on the second light source or the emission wavelength of the fluorescent species rather than from the light originally used to illuminate, and excite, the sample.



Working

Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused on the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.

Forms

The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image. Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).

Typical components of a fluorescence microscope are:

a. Fluorescent dyes (Fluorophore)

A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation. Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.

Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.

b. A light source

Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs.

Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.

c. The excitation filter

The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.

d. The dichroic mirror

A dichroic filter or thin-film filter is a very accurate colour filter used to selectively pass light of a small range of colours while reflecting other colours.

e. The emission filter.

The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.

By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

Advantages of fluorescence microscope:

1. Fluorescence microscopy is the most popular method for studying the dynamic behaviour exhibited in live-cell imaging.
2. This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material.
3. The sensitivity is high enough to detect as few as 50 molecules per cubic micrometer.
4. Different molecules can now be stained with different colours, allowing multiple types of the molecule to be tracked simultaneously.
5. These factors combine to give fluorescence microscopy a clear advantage over other optical imaging techniques, for both in vitro and in vivo imaging.

Limitations of fluorescence microscope:

1. Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence.
2. Cells are susceptible to phototoxicity, particularly with short-wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.
3. Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labelled for fluorescence.

B. Confocal Microscope:

Optical sections are produced in the laser scanning confocal microscope by scanning the specimen point by point with a laser beam focussed in the specimen, and using a spatial filter, usually a pinhole (or a slit), to remove unwanted fluorescence from above and below the focal plane of interest (Fig. 4.11). The power of the confocal approach lies in the ability to image structures at discrete levels within an intact biological specimen. There are two major advantages of using the LSCM in preference to conventional epifluorescence light microscopy. Glare from out-of-focus structures in the specimen is reduced and resolution is increased both laterally in the X and the Y directions (0.14 mm) and axially in the Z direction (0.23 mm).

Image quality of some relatively thin specimens, for example, chromosome spreads and the leading lamellipodium of cells growing in tissue culture (<0.2 mm thick) is not dramatically improved by the LSCM whereas thicker specimens such as fluorescently labelled multicellular embryos can only be imaged using the LSCM.

For successful confocal imaging, a minimum number of photons should be used to efficiently excite each fluorescent probe labelling the specimen, and as many of the emitted photons from the fluorochromes as possible should make it through the light path of the instrument to the detector. The LSCM has found many different applications in biomedical imaging. Some of these applications have been made possible by the ability of the instrument to produce a series of optical sections at discrete steps through the specimen (Fig. 4.12). This series of optical sections collected with a confocal microscope are all in register with each other, and can be merged together to form a single projection of the image (Z projection) or a 3D representation of the image (3D reconstruction).

Multiple-label images can be collected from a specimen labelled with more than one fluorescent probe using multiple laser light sources for excitation (Fig. 4.13, see also colour section). Since all of the images collected at different excitation wavelengths are in register it is relatively easy to combine them into a single multicoloured image. Here any overlap of staining is viewed as an additive colour change. Most confocal microscopes are able to routinely image three or four different wavelengths simultaneously. The scanning speed of most laser scanning systems is around one full frame percentage second. This is designed for collecting images from fixed and brightly labelled fluorescent specimens. Such scan speeds are not optimal for living specimens, and laser scanning instruments are available that scan at faster rates for more optimal live cell imaging. In addition to point scanning, swept field scanning rapidly moves a mm thin beam of light horizontally and vertically through the specimen.

Optical sectioning: Many images collected from relatively thick specimens produced using epifluorescence microscopy are not very clear. This is because the image is made up of the optical plane of interest together with contributions from fluorescence above and below the focal plane of interest. Since the conventional epifluorescence microscope collects all of the information from the specimen, it is often referred to as a wide field microscope. The ‘out-of-focus fluorescence’ can be removed using a variety of optical and electronic techniques to produce optical sections. The term optical section refers to a microscope’s ability to produce sharper images of specimens than those produced using a standard wide field epifluorescence microscope by removing the contribution from out-of-focus light to the image, and in most cases, without resorting to physically sectioning the tissue. Such methods have revolutionised the ability to collect images from thick and fluorescently labelled specimens such as eggs, embryos and tissues. Optical sections can also be produced using high-resolution DIC optics, micro computerised tomography (CT) scanning or optical projection tomography. However, currently by far the most prevalent method is using some form of confocal or associated microscopical approach.

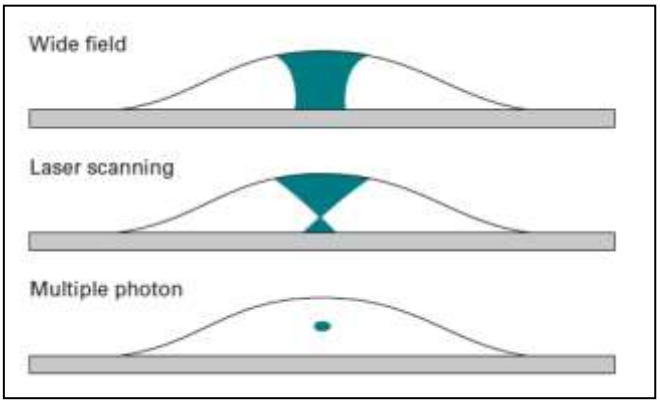


Figure: Illumination in a wide field, a confocal and a multiple photon microscope. The diagram shows a schematic of a side view of a fluorescently labelled cell on a coverslip. The shaded green areas in each cell represent the volume of fluorescent excitation produced by each of the different microscopes in the cell. Conventional epifluorescence microscopy illuminates throughout the cell. In the LSCM fluorescence illumination is throughout the cell but the pinhole in front of the detector excludes the out-of-focus light from the image. In the multiple photon microscope, excitation only occurs at the point of focus where the light flux is high enough.

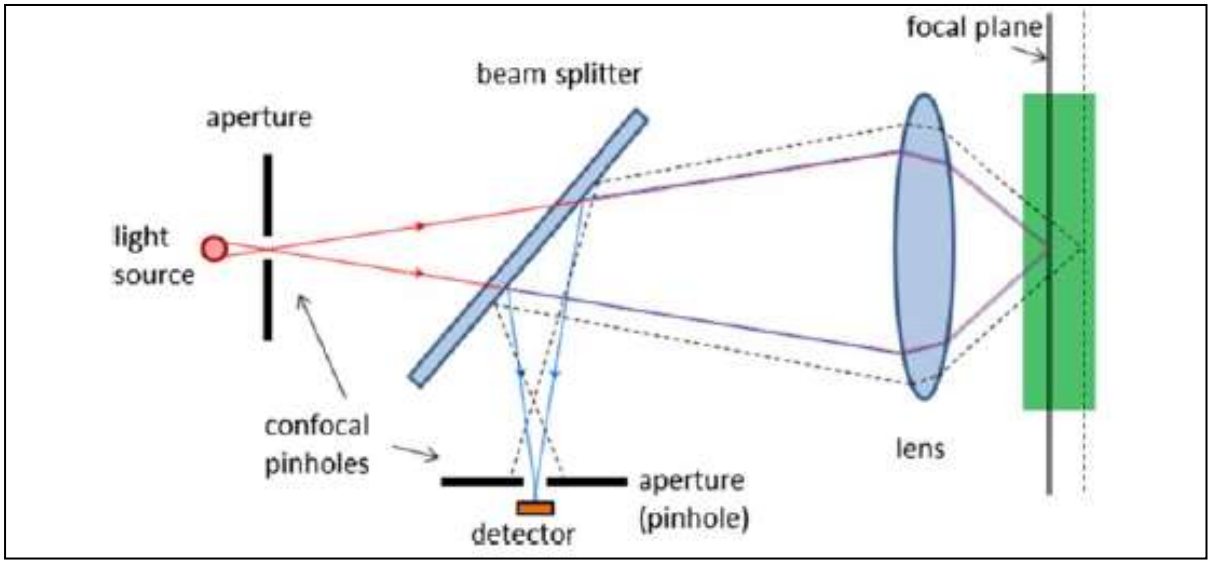


Figure: The principle of confocal microscopy. Only light reflected by structures very close to focal plane can be detected.

C. Phase Contrast Microscope:

In recent years, remarkable advances have been made in the study of living cells (unstained) by the development of special optical techniques such as phase contrast and interference are highly transparent to visible light and they cause phase changes in transmitted radiations microscopy.

The biological specimens.

The phase contrast microscope has the same resolving power as the ordinary light microscope but it permits visualization of different parts of the cell due to differences in their refractive

index (Refractive index is defined as the ratio of the velocity of light in a vacuum to its velocity in a transmitting medium).

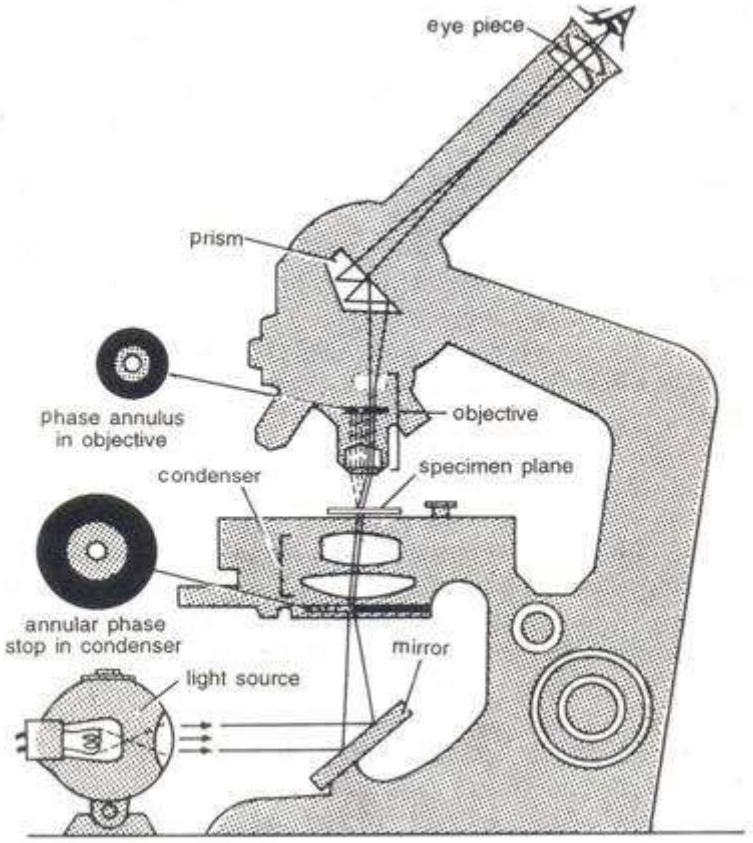


Fig. 2. The light path in a phase contrast microscope.

Because light is transmitted through a structure at a velocity inversely proportional to the refractive index of the structure, light waves emerging from structures with different refractive index will be out of phase with one another. The phase contrast microscope is able to convert these differences in phase to differences in light intensity, producing an image with good contrast. The phase-contrast microscope utilizes interference between two beams of light.

In the phase contrast microscope, the small phase differences are intensified. The most lateral light passing through the objective lens of the microscope is advanced or retarded by an additional l/4th wavelength (1/4λ.) with respect to the central light passing through the medium around the object, by an annular phase plate that introduces a 1/4 wavelength variation in the back focal plane of the objective.

In addition an annular diaphragm is placed in the substage condenser. The phase effect results from the interference between the direct geometric image given by the central part of the objective and the lateral diffracted image, which has been retarded or advanced to a total of 1/2 wavelength. In bright or negative contrast, the two sets of rays are added and the object appears brighter than the surroundings. In dark or positive contrast, the two sets of rays are subtracted making the image of the object darker than the surroundings. Because of this interference, the minute phase changes within the object are amplified and intensified.

A transparent object thus appears in various shades of gray, depending upon the thickness of the object and the difference between the refractive indices of the object and the medium. Phase microscopy is used to observe living cells and tissues. It is particularly valuable for observing the cells cultured in vitro during mitosis. In addition an annular diaphragm is placed in the

substage condenser. The phase effect results from the interference between the direct geometric image given by the central part of the objective and the lateral diffracted image, which has been retarded or advanced to a total of 1/2 wavelength. In bright or negative contrast, the two sets of rays are added and the object appears brighter than the surroundings. In dark or positive contrast, the two sets of rays are subtracted making the image of the object darker than the surroundings. Because of this interference, the minute phase changes within the object are amplified and intensified.

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Principle of the phase-contrast microscope:

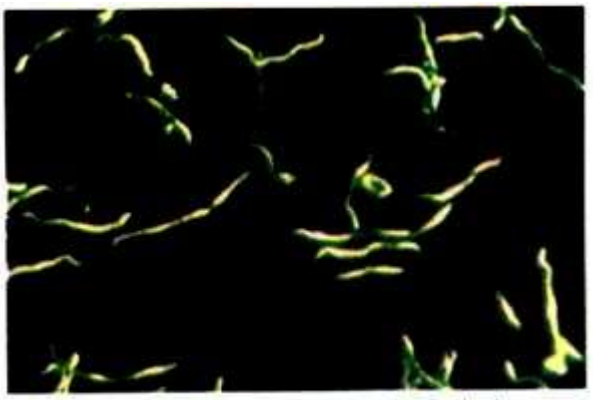
This is to convert small phase differences into differences in contrast that can be detected visually. An annular phase plate is placed in the objective of the microscope and an annular diaphragm is placed in the condenser as shown in the figure 2. As light is transmitted through the lenses, some of the rays pass through in a direct path while others are diffracted laterally. Diffracted light rays are, thus out of phase with the direct light, and an image of strong contrasts is produced. The annular diaphragm illuminates the object with a narrow cone of light, and the annular phase plate produces a variation of 1/4 A. between the diffracted lateral light and the direct light. The phase effect is the result of interference between the direct image in the centre of the objective and the diffracted lateral image. If the diffracted image is retarded, negative contrast results, whereas if it is advanced, positive contrast results. When the refractive index of the medium is greater than that of the object, the object is dark, and when the refractive index of the medium is less than that of the object, the object is bright.

D, Dark Field Microscope:

In this type of microscopy, a dark back ground is produced against which objects are brilliantly illuminated. For this purpose the light microscope is equipped with a special kind of condenser that transmits a hollow core of light from the source of illumination. Thus, if the aperture of condenser is allowed to open completely, and a dark field stop inserted below the condenser, the light rays reach the objects form a hollow core.

Any object within this beam of light will reflect some light into the objective and will be visible. This method of illuminating an object where the object appears self-illuminous against a dark field, called dark-field illustration.

The condensers used are Abbe condenser, paraboloid condenser and cardoid condenser. Dark field microscopy is particularly valuable for the examination of unstained microorganisms suspended in fluid wet mount and hanging drop operations.



Treponema pallidum, the spirochete that causes syphilis; dark-field microscopy.

Electron Microscopy:

In 1931 Knoll and Ruska, German scientists discovered electron microscopy. Von Borries and Ruska (1938) in Berlin constructed first practical electron microscope. The commercial instrument first came in around 1940.

In electron microscope the source of illumination is electron beam. The construction and principle of electron microscope are easily related to those of light microscope. The range of wave length of visible light used in light microscope is 4000 Å – 7800 Å, while with an electron microscope employing 60-80 KV electron, the wave length is only 0.05 Å.

In the instrument as shown in the figure, the electron gun generates electron beam. These electrons are concentrated by other components of electron gun producing a fast moving narrow beam of electron.

Electrons are focused by electromagnetic lenses. Electromagnetic lens consists of wire encased in soft iron casing. When electric current is passed through the coil, it generates an electromagnetic field through which electrons are focused.

There are three general types of electromagnetic lenses. The one is placed between the source of illumination and the specimen. This focuses the beam of electron on specimen functions in a similar manner as that of light microscope. The other two lenses are on the opposite side of specimen which magnify the image in similar fashion as objective and ocular in light microscope.

Construction of an Electron Microscope:

An electron microscope consists of an electric gun, microscope column, electromagnetic coils, a fluorescent screen and some other accessories described below:

(a) The electron gun is located at the top of the body of microscope. It is the source of electrons. It is made up of a tungsten filament surrounded by a negatively biased shield with an aperture. The electron beam is drawn off through this aperture.

(b) The microscope column or central column is made up of an evacuated metal tube. It protects the person operating the microscope from X-rays that are generated when the electrons strike the surface of the metal tube.

(c) The electromagnetic coils or lenses include projector coils, objective arid condenser. In each coil, the coils of electric wire are wound on a hollow metallic cylinder. The magnetic

field, produced by passing the electric current through the magnetic coil, functions as a magnifying lens.
(d) The fluorescent screen is used for observing the magnified image of the object. It remains coated with a chemical which, on being excited, forms the image as on the screen of television.
(e) Some other essential accessories of the electron microscope include high voltage transformers (for developing high voltage current for the electron gun and electromagnetic coils), vacuum pumps (for maintaining high vacuum inside the microscope column), a water cooling system (for prevention from overheating of various parts), a circulating pump, a refrigeration plant and also a filter system.

All these parts require elaborate arrangements and contribute to the massive size of the electron microscope. The image formation in this microscope occurs by the scattering of electrons. The electrons strike the atomic nuclei and get dispersed. These dispersed electrons form the electron image. By projecting on a fluorescent screen or photographic film, this electron image is converted into a visible image of the object. The electron beam in this microscope is made by accelerating electrons through a potential difference of from 1-1500 kV in an electron gun.
Only dried specimens are studied by electron microscope. Living cells cannot be studied with this microscope because they possess water which causes large scale scattering of electrons.
Ultrathin sections (10-50 nm thickness), which are more than 200 times thinner than those routinely used for light microscopy, are cut for electron microscopy. These are cut with the help of diamond or glass knives of an ultra-microtome.

Electron microscopes are of two types:
(1) Transmission electron microscope, and (
2) Scanning electron microscope.

a. Transmission Electron Microscope:
This microscope forms an image of the specimen by the electrons that have passed through the specimen (Fig. 21.2). The components of the specimen that scatter electrons appear dark and are called **"electrons dense".** The part that have less ability of electron scattering appear light. The electron scattering ability of the element with higher atomic number, such as, uranium, lead etc. is greater than those of lower atomic numbers. The biological molecules are composed of the elements with comparatively low atomic number, viz., hydrogen, carbon, nitrogen, oxygen, phosphorus and sulphur. These elements have poor electron scattering ability. Therefore, biological molecules are stained with metals of high atomic number such as uranium, lead and osmium.
The material is fixed in osmium tetrachloride, $KMnO_4$ or phosphotungstic acid. The fixed tissues are then embedded in hard plastic resin. Ultra microtome is used to cut ultrathin sections (50-100 nm) of the material. These section are examined under the electron microscope. Intact organelles and viruses are not sectioned. Followings are some techniques used to observe the materials by electron microscope.

Shadow Casting:
This technique is used to un-sectioned materials e.g., viral particles. The sample dried on a film supported by a grid is placed in an evaporation chamber. The chamber is evacuated. Heavy

metal atoms projected from a glowing filament impinge at a predetermined angle on the film (Fig. 21.4).

The metal is deposited as a uniform electron opaque layer on the film. The metal is deposited on one side of the specimen, while the other side lacks the deposition.

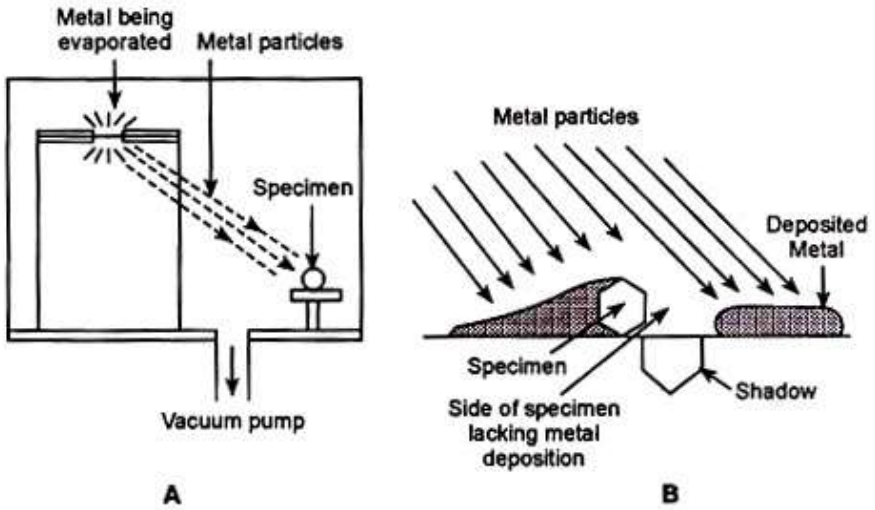


Fig. 21.4. Diagram showing method of shadow casting. **A.** Evaporation chamber. **B.** Shadow of specimen under electron microscope.

Examination under the electron microscope shows the "shadow" of the specimen in the place lacking the deposited metal. The size and shape of shadow provide the information on the 3-dimensional shape of the material.

Positive Staining:

The specimen is stained and than the excess of stain is removed. It gives an unstained background and stained object. Certain viruses can be stained by salts that become absorbed selectively. For example uranyl acetate stains the viral nucleic acid and other components. Abs conjugated to ferritin (electron opaque molecule) stains the protein.

Negative Staining:

Negative staining can be used to study the viral particles and organelles. The viral particles are mixed with salt, such as sodium phosphotungstate which is highly opaque to electrons. The mixture is spread on a carbon membrane and dried.

The regions of the particles which are not penetrated by salt form electron lucent area on an opaque background. Details of the surface structure is revealed by perpetration between protruding parts of the salt.

The Whole Mount Technique:

This technique is also used for the un-sectioned materials, but it does not involve staining or heavy metal deposition. The scattering of electrons from the object produces the image.

The Freeze-etch Technique:

By this technique, a unique picture of cells is viewed, especially where the membrane is involved. The cell is broken along and across the membranes and therefore, it shows the four

views of the biological membrane, viz., protoplasmic surface, exoplasmic surface, protoplasmic fracture faces, and exoplasmic fracture faces.
The technique does not involve fixatives, stains and embedding agents and therefore, the cell structure is not deformed.

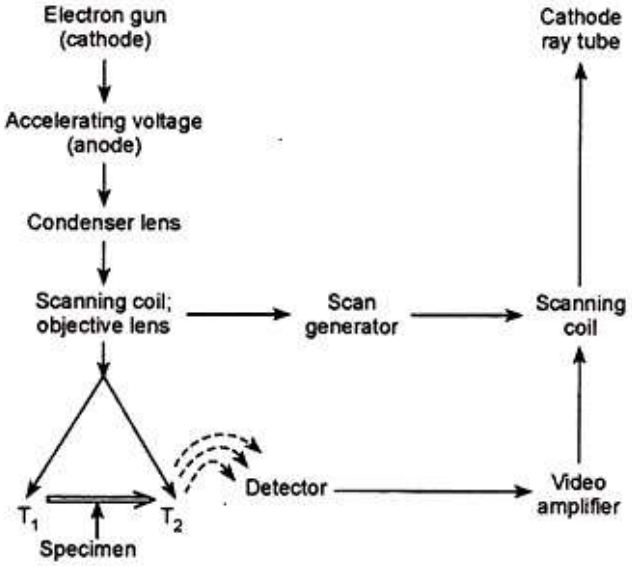


Fig. 21.5. Schematic view of the working system of a scanning electron microscope. The electron beam illuminates different points in the specimen at different times, T_1 and T_2. Movement of this beam is synchronized with beam in cathode ray tube by a scan generator. Detector picks the scattered electrons from specimen and modulates the beam in the cathode ray tube; an the scattered electrons from specimen and modulates the beam in the cathode ray tube; an image of the specimen is formed.

The material to be studied is frozen in liquid Freon in a vacuum. The cell function is instantly arrested due to rapid freezing. The frozen material becomes very hard, and when struck by a knife, it is broken along the lines of membranes.
Water is evaporated by placing the broken material in a vacuum. Water loss causes the "etching effect" i.e., details become much clearer. A heavy metal (e.g., platinum) is used for shadowing the fractured surface, and a replica is prepared by using a carbon film. A strong acid is used to remove the tissue and to leave the metal replica. This metal replica is viewed with the electron microscope.

b. Scanning Electron Microscope:
Scanning electron microscopes combine the mechanism of electron microscopy and television. SEM became commercially available in early 1960's and the researchers were Knoll, Von Ardenne, Zworytein etc.
In SEM, electrons are not transmitted through the very thin specimen from below but impinge on its surface from above. The specimen may be opaque and of any manageable thickness and size. If the specimen is an electron conductor, it needs only to be held on an appropriate support. If it is non-conductor, it is allowed to dry but if moist, freeze dried in liquid nitrogen is necessary. The specimen is then coated with metal vapour (gold) in vacuum. The electrons originate at high energy (20,000 V) from a hot tungsten or lanthanum hexoboride cathode "gun". These electrons are sharply focused, adjusted and narrowed by an arrangement of magnetic fields.

Instead of forming a broad inverted cone of rays, in SEM a needle sharp probe (about 5 – 10 mm in diameter) is made. This primary beam (probe) acts only as an exciter of image forming secondary electrons emerging from the surface of the specimen.

The probe scans the specimen like that on a blank TV screen. The probe can impinge on depth and heights with equal speed and accuracy giving great depth of field and producing images with three dimensions. Images are elicited from wherever the probe strikes the metal coated areas of the specimen. Magnification is the ratio of final image to the diameter of area scanned.

Any of the secondary electrons with sufficient energy can emerge from the surface. Those that emerge not too far from the point of impact of the probe can be used to form an image. The useful secondary electrons are magnetically deflected to a collector or detector. Here, they produce a signal that represents at any single moment, only 5-10 mm area or spot of impingement of the probe on the specimen.

The successive signals from the collector are amplified and transmitted to a cathode ray (TV) tube. The scanning beam and TV tube beam are synchronized.

The image scan by the eye on TV screen is thus the sequence of signals representing in araster pattern, the successive areas traversed by the primary probe beam. Exposure may range from a few second to one-half hour or more. The TV image may be photographed, video taped or processed in motion on a computer.

This microscope shows 3-dimensional surface architecture of cells and organelles. The present day scanning electron microscopes have the resolution power of 10 nm which is less than the resolution power of transmission electron microscopes.

However, this resolving power can be increased by making further improvements. In this system (Fig. 21.5), a beam of electrons is used that moves back and forth across the specimen by a canning coil.

It illuminates different points on the surface of specimen at different times. The scan generator synchronizes the movement of this beam in a cathode ray rube (television tube). Electrons are deflected from the specimen and are picked up by a detector that modulates the beam in the cathode ray tube. A 3-dimensional structure of the surface of the cell or organelle is obtained.

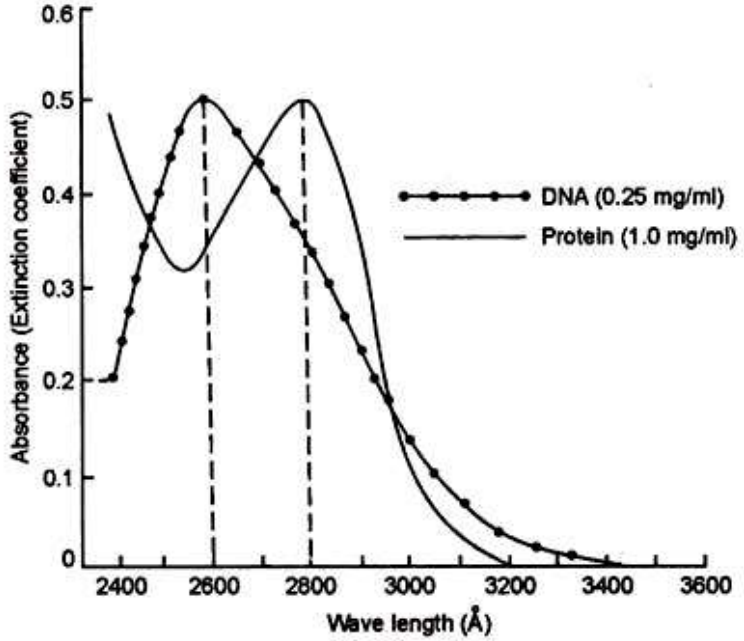


Fig. 21.6. Degree of ultraviolet absorption by DNA and proteins at different wavelengths (10Å = 1 nm).

Difference : Light Microscope vs electron Microscope:

Light Microscope :

1. Visible light is used in this microscope.
2. Source of illumination is situated at the bottom.
3. For magnification in this microscope the lens system consists of glass lenses.
4. The lenses are ocular, objective and condenser
5. The image is either seen with the eye or recorded on a photographic film with a camera in this microscope.

Electron Microscope:

1. Electrons are used in this microscope.
2. Source of illumination is situated at the top in this microscope.
3. The lens system consists of electromagnetic coils in this microscope.
4. This microscope has projector coils, an objective and a condenser.
5. The image in an electron microscope is either recorded on a fluorescent scent screen or recorded on a photographic film.

Difference between TEM and SEM:

Both SEM (scanning electron microscope/microscopy) and TEM (transmission electron microscope/microscopy) refer both to the instrument and the method used in electron microscopy.

There are a variety of similarities between the two. Both are types of electron microscopes and give the possibility of seeing, studying, and examining small, subatomic particles or compositions of a sample. Both also use electrons (specifically, electron beams), the negative charge of an atom. Also, both samples in use are required to be "stained" or mixed with a particular element in order to produce images. Images produced from these instruments are highly magnified and have a high resolution.

However, an SEM and TEM also share some differences. The method used in SEM is based on scattered electrons while TEM is based on transmitted electrons. The scattered electrons in SEM are classified as backscattered or secondary electrons. However, there is no other classification of electrons in TEM.

The scattered electrons in SEM produced the image of the sample after the microscope collects and counts the scattered electrons. In TEM, electrons are directly pointed toward the sample. The electrons that pass through the sample are the parts that are illuminated in the image. The focus of analysis is also different. SEM focuses on the sample's surface and its composition. On the other hand, TEM seeks to see what is inside or beyond the surface. SEM also shows the sample bit by bit while TEM shows the sample as a whole. SEM also provides a three-dimensional image while TEM delivers a two-dimensional picture.

In terms of magnification and resolution, TEM has an advantage compared to SEM. TEM has up to a 50 million magnification level while SEM only offers 2 million as a maximum level of

magnification. The resolution of TEM is 0.5 angstroms while SEM has 0.4 nanometers. However, SEM images have a better depth of field compared to TEM produced images. Another point of difference is the sample thickness, "staining," and preparations. The sample in TEM is cut thinner in contrast to a SEM sample. In addition, an SEM sample is "stained" by an element that captures the scattered electrons.

In SEM, the sample is prepared on specialized aluminium stubs and placed on the bottom of the chamber of the instrument. The image of the sample is projected onto the CRT or television-like screen.

On the other hand, TEM requires the sample to be prepared in a TEM grid and placed in the middle of the specialized chamber of the microscope. The image is produced by the microscope via fluorescent screens.

Another feature of SEM is that the area where the sample is placed can be rotated in different angles. TEM was developed earlier than SEM. TEM was invented by Max Knoll and Ernst Ruska in 1931. Meanwhile, SEM was created in 1942. It was developed at a later time due to the complexity of the machine's scanning process.

Flow Cytometry:

Basic principles of flow cytometry

Flow cytometry is used when there is a need to profile a large number of different cell types in a population. The cells are separated on the basis of differences in size and morphology. Additionally, fluorescently-tagged antibodies that target specific antigens on the cell surface can be used to identify and segregate various sub-populations.

The basic steps include passing the cells through a narrow channel, such that each cell is illuminated by a laser one at a time. A series of sensors then detect the refracted or emitted light, and this data is integrated and compiled to generate information about the sample.

Flow cytometry principle - How the fluorescence activated cell sorting (FACS) work ?

Flow cytometry is a technique to identify and isolate cells from a mixture of other cells using fluorescence activity. Flow cytometry was developed by Fulwyler in 1965. Till today it is used for research in cell biology. In that technique cell sorting and cell counting was done by using laser light technology.

There are different steps involved in a process of flow cytometer; First step is Flow of cell in that liquid containing cells i.e. liquid stream is passing single file through light beam of laser light for sensing. Second step is that measuring system, which commonly used for measurement of conductivity, Optical system containing Mercury and Xenon lamp resulting in light signal. The third step is to detection of light scattering, in that step light signal are converted analogue to digital signal with the help of Analogue to digital conversion system. It will detect Forward scatter light (FSC) and Side scatter light as well as fluorescence signal from light in to electrical signal that can be processed by computer. The fourth step is that analysis of signal by computer, in that collecting of data from sample using cytometer this collecting of data is termed as Acquisition. This acquisition is carried out by computer connected to the flow cytometer software. This software handle the digital interface with cytometer, it is able to adjusting the parameter required for the voltage compensation. It is also

monitor initial sample analysis. Fluorescence labelled antibodies was developed for clinical research. In modern instrument contain multiple laser and fluorescence detector, currently in industrial instrument ten laser and 18 fluorescence detector. More number of detector and laser allow for multiple antibody labelling and identify a target population by their marker. In certain instrument can even take digital image of individual cell for the analysis.

The data is to be analysed by data generated by computer either in histogram or dot plot. Computer analysis give automated population identification, this automated identification could potentially help finding of rare hidden population. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. The technique was expanded by Len Herzenberg, who was responsible for coining the term FACS.

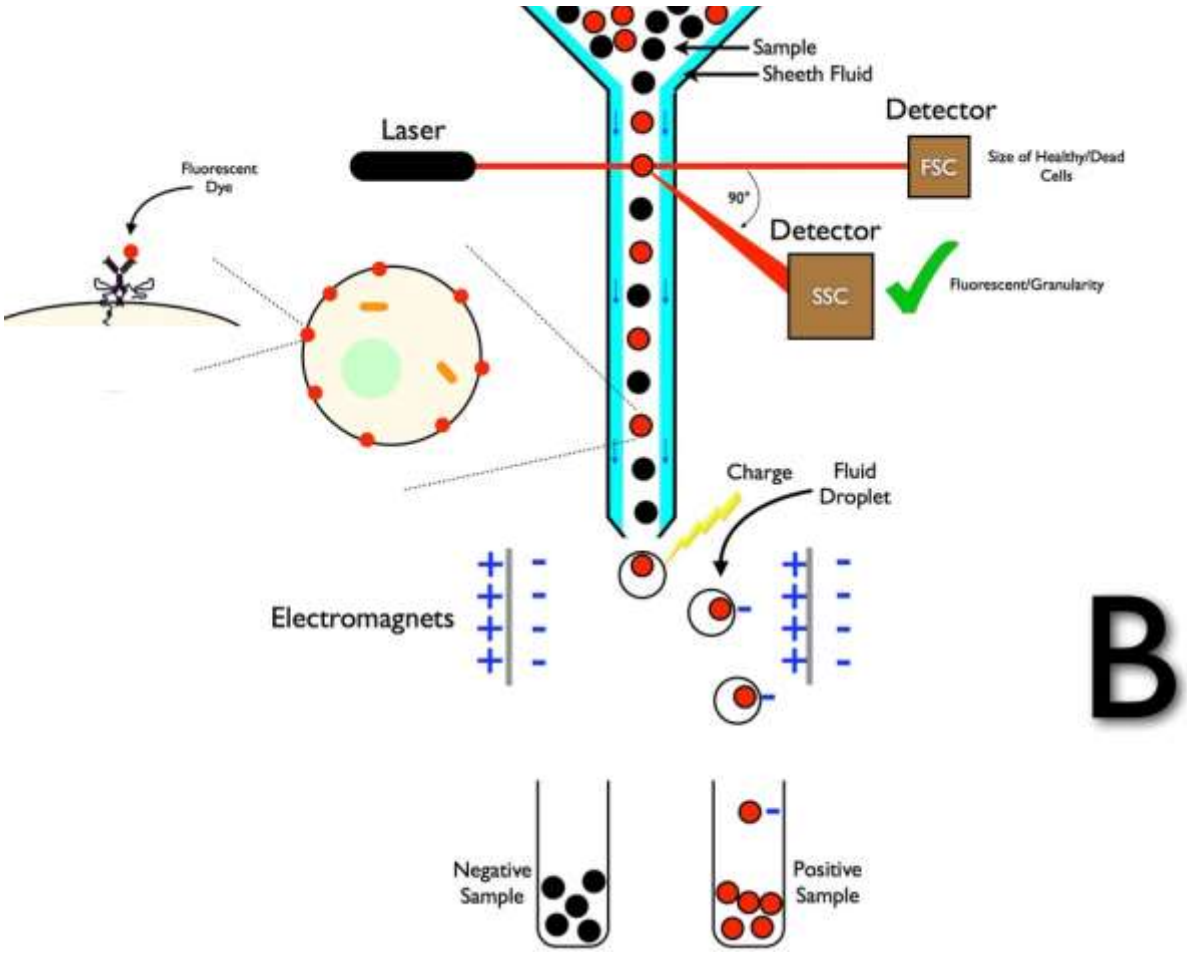


Fig- Fluorescence-Activated Cell Sorting (FACS) principle

The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is

adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off

The technology has applications in a number of fields, including molecular biology, pathology, immunology, plant biology and marine biology It has broad application in medicine (especially in transplantation, hematology, tumor immunology and chemotherapy, prenatal diagnosis, genetics and sperm sorting for sex preselection). Also, it is extensively used in research for the detection of DNA damage, caspase cleavage and apoptosis In marine biology, the autofluorescent properties of photosynthetic plankton can be exploited by flow cytometry in order to characterise abundance and community structure. In protein engineering, flow cytometry is used in conjunction with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties

What can flow cytometry be used to measure?

Flow cytometry helps to analyse several parameters of a cell simultaneously. Some of these parameters are described below:

Functional analysis

This method can determine biological activity inside cells, such as the generation of reactive oxygen species, mitochondrial membrane changes during apoptosis, phagocytosis rates in labelled bacteria, native calcium content, and changing metal content in response to drugs, etc.

Determining cell viability

This method can also be used to assess cell viability after the addition of pathogenic organisms or drugs. Any breach in cell membrane integrity can be determined using dyes that can enter the punctured cell membrane. Fluorescent probes such as bis-oxonol can bind to proteins present on the cell membrane, allowing for the identification of various stages of necrosis.

Measuring apoptosis and necrosis

Apoptosis or programmed cell death is accompanied by characteristic changes in cell shape, loss of structures, cell detachment, condensation of the cytoplasm, cell shrinkage, phagocytosis of cellular residues and changes in the nuclear envelope.

Some of the biochemical changes include proteolysis, DNA denaturation, cell dehydration, protein cross-linking, and a rise in the free calcium ions. These physical and biochemical changes can be detected using flow cytometry.

Oncosis is a necrotic event where the cell starts to swell rather than shrink. This leads to rupture of the plasma membrane and release of proteolytic enzymes that can also damage the surrounding tissues. These changes in the plasma membrane and cell shape can be assessed using flow cytometry.

Cell cycle analysis:

The amount of DNA present in the nucleus varies during each phase of the cell cycle. This variation in DNA content can be assessed using fluorescent dyes that bind to DNA or monoclonal antibodies, which can allow the detection of antigen expression.

Other factors including the content of cell pigments such as chlorophyll, DNA copy number variation, intracellular antigens, enzymatic activity, oxidative bursts, glutathione, and cell adherence can similarly be measured using this method.

Data interpretation

Each cell that passes through the laser light is detected as a separate event. Also, different types of detected light: forward-scatter, side-scatter, and specific wavelengths of fluorescence emission, is assigned a distinct channel. The data for each of these events is plotted independently and can be represented by two methods: histograms and dot-plots.

Histograms compare a single parameter, where intensity is plotted on one axis and the number of events is plotted on a separate axis. Dot-plots can compare more than one parameter simultaneously, where each event is displayed as a single point and the intensity values of two or three channels are represented on the various axis.

In this scenario, events that have similar intensities cluster together in the dot plot. While dot-plots can compare multiple parameters together, histograms are easier to read and understand. In many cases, dot-plots and histograms are not mutually exclusive, and in many flow cytometry experiments both types of graphs are plotted to represent and assess multi-parametric data.

Probable Questions:

1. Write the basic principle of fluorescent microscope?
2. Describe different components of fluorescent microscope.
3. What are the advantages of fluorescent microscope ?
4. What are the limitations of fluorescent microscope ?
5. Describe basic principle of phase contrast microscope.
6. Describe basic principle of Dark field microscope.
7. Describe basic principle of confocal microscope.
8. Describe basic components of a electron microscope.
9. What is shadow casting in electron microscopy?
10. What is positive and negative staining in electron microscopy?
11. Write five differences between light microscope and electron microscope.
12. Differentiate between TEM and SEM.
13. Describe the working principle of FACS with suitable diagram.
14. Write different utility of FACS.

Suggested Readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7^{th} Edition.
2. Biophysical Chemistry by Upadhyay and Upadhyay.

Unit-VIII
Cell fractionation methods:
a) Preparative Ultracentrifugation b) Gradient Centrifugation

Objective: In this unit we will discuss different cell fractionation methods such as preparative ultracentrifugation and gradient centrifugation

Basic Principle of Centrifugation Technique:
The principle of the centrifugation technique is to separate the particles suspended in liquid media under the influence of a centrifugal field. These are placed either in tubes or bottles in a rotor in the centrifuge. Particles differing in sizes, shape and density are separated as their sedimentation rate is different.

The centrifugal force is generated by rotating the rotor of the centrifuge at a high speed. Besides normal and high speed centrifuge there is a very high speed centrifuge known as Ultracentrifuge, which is developed by Theodor Svedberg in 1940.

This instrument is designed to produce centrifugal forces up to several hundred thousand times which can separate and purify subcellular organelles, proteins, nucleic acids and several macromolecules. Thus the ultracentrifuge has opened up a new line in many types of fundamental studies in Cell Biology, Biochemistry and Molecular Biology.

The rate of sedimentation of a particle in a centrifugal force can be shown in the following:

$$\frac{dx}{dt} = \frac{2r^2(P_p - P_m)}{gn} \qquad (1)$$

where $\frac{dx}{dt}$ = the rate at which the particle moves towards the centrifugal field
r = radius of the particle
P_p = densities of the particle
P_m = densities of the medium
n = viscosity of the suspending medium
g = centrifugal force

Now, the particles will not move if the densities of the particles and the medium are equal. If the densities of the particles are greater than the medium, they will move toward the bottom of the centrifuge tube while they will remain at the top of the tube, if the particles are lighter than the medium.

The centrifugal force produced by the centrifuge is measured by the gravity units as:

$$G = w^2 r \qquad (2)$$

where G = centrifugal field
w = angular velocity of the rotor
r = radial distance of the particle from the axis of the rotation

Again, angular velocity of the rotor is known as in the formula:

$$w = \frac{2\text{rev/min}}{60} \qquad (3)$$

Putting the value of w in the equation (2), the centrifugal field (G) will be

$$G = \frac{4^2\ (\text{revolution/min})^2\ r}{60 \times 60}$$

$$\therefore\ G = \frac{4^2\ (\text{rev/min})^2\ r}{3,600}$$

Which is generally shown as a multiple of g, i.e., gravitational field of the earth. Sometimes it is also expressed as R.C.F (Relative Centrifugal Field) which is the ratio of the weight of the particle in the centrifugal field to the weight of the same particle acted on by gravity. On the basis of this principle of separation, particles are separated depending on their densities, size, centrifugal force, time of separation etc. Different cell components are separated in the following order—whole cells and cell debris first followed by nuclei, plastids, mitochondria, lysosomes, microsomes, fragments of endoplasmic reticulum and ribosome.

The method of separation becomes complicated when the particles are not spherical, which requires some complicated formula for calculation. In case of Ordinary rotors as used in the preparative centrifuge, the centrifugal field does not remain uniform, because the radial dimension of a particle will vary according to the position in the centrifuge tube (r_{min} and r_{max}). The particle will have a greater centrifugal field as it is further away from the axis of rotation. This occurs both in the fixed angle and swing- away rotor (Fig. 7.1). Hence, the centrifugal field is calculated from the average radius of rotation (r_{av}.) of the column of liquid in the centrifuge tube. The details of maximum and the method of calculation of relative centrifugal field (R.C.F.) are generally given in the manual of the centrifuge.

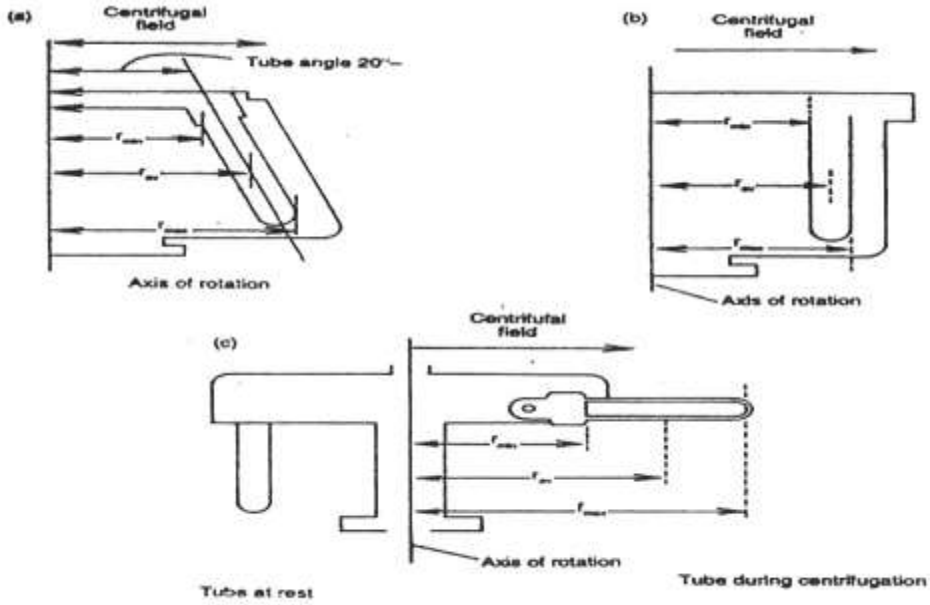


Fig. 7.1: Diagrammatic representation of: (a) fixed angle; (b) vertical tube and (c) swinging bracket.

The sedimentation rate of a particle can also be expressed as sedimentation coefficient (s) which is the sedimentation rate per unit of centrifugal field. The sedimentation values depend on the solvent-solute systems. As the sedimentation coefficient of many of macromolecules is very small, the basic unit is taken as $10^{\sim 13}$ seconds and is designated as Svedberg unit (S). For example, the Ribosomal RNA showing sedimentation values as 5 x 106^{-13} seconds is said to be 5S (5 Svedberg units).

Sedimentation coefficients of some of the macromolecules are shown in Table 7.1.

Table 7.1: Sedimentation coefficients of some macromolecules

Macromolecules	Range of sedimentation coefficients in S
Soluble proteins	2 to 25 S
Nucleic acids	5 to 100 S
Ribosome	20 to 200 S
Viruses	40 to 1,000 S
Lysosomes	4,000 S
Mitochondria	20×10^3 S to 70×10^3 S
Nuclei	$4,000 \times 10^3$ S to $40,000 \times 10^3$ S

Preparative centrifugation:

Cellular and subcellular fractionation techniques are indispensable methods used in biochemical research. Although the proper separation of many subcellular structures is absolutely dependent on preparative ultracentrifugation, the isolation of large cellular structures, the nuclear fraction, mitochondria, chloroplasts or large protein precipitates can be achieved by conventional high-speed refrigerated centrifugation. Differential centrifugation is

based upon the differences in the sedimentation rate of biological particles of different size and density. Crude tissue homogenates containing organelles, membrane vesicles and other structural fragments are divided into different fractions by the stepwise increase of the applied centrifugal field. Following the initial sedimentation of the largest particles of a homogenate (such as cellular debris) by centrifugation, various biological structures or aggregates are separated into pellet and supernatant fractions, depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles. To increase the yield of membrane structures and protein aggregates released, cellular debris pellets are often rehomogenised several times and then recentrifuged. This is especially important in the case of rigid biological structures such as muscular or connective tissues, or in the case of small tissue samples as is the case with human biopsy material or primary cell cultures.

The differential sedimentation of a particulate suspension in a centrifugal field is diagrammatically shown in Fig. 3.4a. Initially all particles of a homogenate are evenly distributed throughout the centrifuge tube and then move down the tube at their respective sedimentation rate during centrifugation. The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant. However, during the initial centrifugation step smaller particles also become entrapped in the pellet causing a certain degree of contamination. At the end of each differential centrifugation step, the pellet and supernatant fraction are carefully separated from each other. To minimise cross-contamination, pellets are usually washed several times by resuspension in buffer and recentrifugation under the same conditions. However, repeated washing steps may considerably reduce the yield of the final pellet fraction, and are therefore omitted in preparations with limiting starting material. Resulting supernatant fractions are centrifuged at a higher speed and for a longer time to separate medium-sized and small-sized particles. With respect to the separation of organelles and membrane vesicles, crude differential centrifugation techniques can be conveniently employed to isolate intact mitochondria and microsomes.

Density-gradient centrifugation:

To further separate biological particles of similar size but differing density, ultracentrifugation with preformed or self-establishing density gradients is the method of choice. Both rate separation or equilibrium methods can be used. In Fig. 3.4b, the preparative ultracentrifugation of low- to high-density particles is shown. A mixture of particles, such as is present in a heterogeneous microsomal membrane preparation, is layered on top of a preformed liquid density gradient. Depending on the particular biological application, a great variety of gradient materials are available. Caesium chloride is widely used for the banding of DNA and the isolation of plasmids, nucleoproteins and viruses. Sodium bromide and sodium iodide are employed for the fractionation of lipoproteins and the banding of DNA or RNA molecules, respectively.

Various companies offer a range of gradient material for the separation of whole cells and subcellular particles, e.g. Percoll, Ficoll, Dextran, Metrizamide and Nycodenz. For the separation of membrane vesicles derived from tissue homogenates, ultra-pure DNase, RNase and protease-free sucrose represents a suitable and widely employed medium for the preparation of stable gradients. If one wants to separate all membrane species spanning the whole range of particle densities, the maximum density of the gradient must exceed the density

of the most dense vesicle species. Both step gradient and continuous gradient systems are employed to achieve this. If automated gradient makers are not available, which is probably the case in most undergraduate practical classes, the manual pouring of a stepwise gradient with the help of a pipette is not so time-consuming or difficult. In contrast, the formation of a stable continuous gradient is much more challenging and requires a commercially available gradient maker. Following pouring, gradients are usually kept in a cold room for temperature equilibration and are moved extremely slowly in special holders so as to avoid mixing of different gradient layers. For rate separation of subcellular particles, the required fraction does not reach its isopycnic position within the gradient. For isopycnic separation, density centrifugation is continued until the buoyant density of the particle of interest and the density of the gradient are equal.

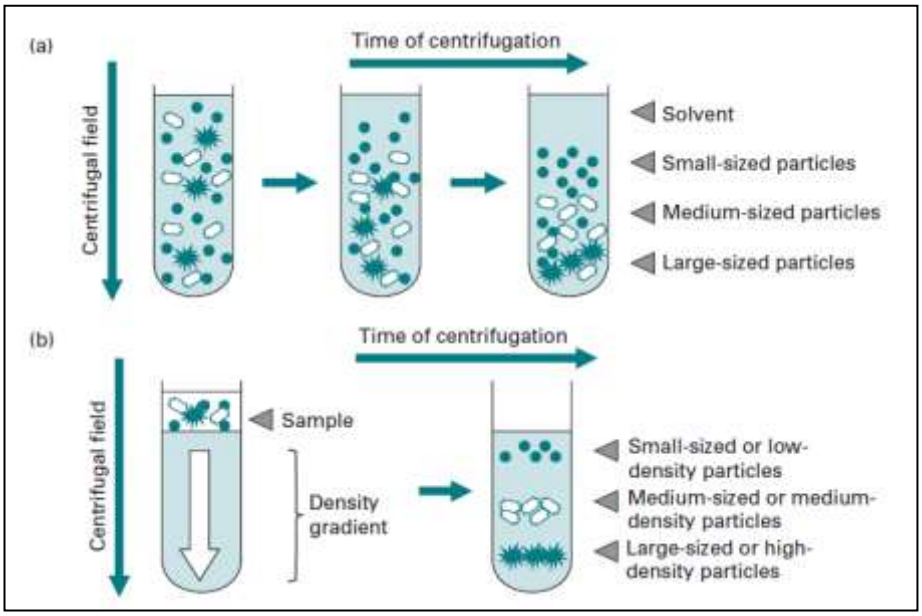


Fig. 3.4 Diagram of particle behaviour during differential and isopycnic separation. During differential sedimentation (a) of a particulate suspension in a centrifugal field, the movement of particles is dependent upon their density, shape and size. For separation of biological particles using a density gradient (b), samples are carefully layered on top of a preformed density gradient prior to centrifugation. For isopycnic separation, centrifugation is continued until the desired particles have reached their isopycnic position in the liquid density gradient. In contrast, during rate separation, the required fraction does not reach its isopycnic position during the centrifugation run.

Practical applications of preparative centrifugation:

To illustrate practical applications of differential centrifugation, density gradient ultracentrifugation and affinity methodology, the isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density is described, the isolation of highly purified sarcolemma vesicles outlined, and the sub-fractionation of liver mitochondrial membrane systems shown. Skeletal muscle fibres are

highly specialised structures involved in contraction and the membrane systems that maintain the regulation of excitation–contraction coupling, energy metabolism and the stabilisation of the cell periphery are diagrammatically shown in Fig. 3.5a. The surface membrane consists of the sarcolemma and its invaginations, the transverse tubular membrane system. The transverse tubules may be subdivided into the non-junctional region and the triad part that forms contact zones with the terminal cisternae of the sarcoplasmic reticulum. Motor neuron-induced depolarisation of the sarcolemma travels into the transverse tubules and activates a voltage-sensing receptor complex that directly initiates the transient opening of a junctional calcium release channel. The membrane system that provides the luminal ion reservoir for the regulatory calcium cycling process is represented by the specialised endoplasmic reticulum. It forms membranous sheaths around the contractile apparatus whereby the longitudinal tubules are mainly involved in the uptake of calcium ions during muscle relaxation and the terminal cisternae provide the rapid calcium release mechanism that initiates muscle contraction. Mitochondria are the site of oxidative phosphorylation and exhibit a complex system of inner and outer membranes involved in energy metabolism.

For the optimum homogenisation of tissue specimens, mincing of tissue has to be performed in the presence of a biological buffer system that exhibits the right pH value, salt concentration, stabilising co-factors and chelating agents. The optimum ratio between the wet weight of tissue and buffer volume as well as the temperature (usually 4 ^{o}C) and presence of a protease inhibitor cocktail is also essential to minimize proteolytic degradation. Prior to the 1970s, researchers did not widely use protease inhibitors or chelating agents in their homogenisation buffers. This resulted in the degradation of many high-molecular-mass proteins. Since protective measures against endogenous enzymes have been routinely introduced into subcellular fractionation protocols, extremely large proteins have been isolated in their intact form, such as 427 kDa dystrophin, the 565 kDa ryanodine receptor, 800 kDa nebulin and the longest known polypeptide, of 2200 kDa, named titin. Commercially available protease inhibitor cocktails usually exhibit a broad specificity for the inhibition of cysteine proteases, serine-proteases, aspartic-proteases, metallo-proteases and amino-peptidases. They are used in the micromolar concentration range and are best added to buffer systems just prior to the tissue homogenisation process. Depending on the half-life of specific protease inhibitors, the length of a subcellular fractionation protocol and the amount of endogenous enzymes present in individual fractions, tissue suspensions might have to be replenished with a fresh aliquot of a protease inhibitor cocktail. Protease inhibitor kits for the creation of individualised cocktails are also available and consist of substances such as trypsin inhibitor, E-64, aminoethyl-benzenesulfonyl fluoride, antipain, aprotinin, benzamidine, bestatin, chymostatin, E-aminocaproic acid, N-ethylmaleimide, leupeptin, phosphoramidon and pepstatin. The most commonly used chelators of divalent cations for the inhibition of degrading enzymes such as metallo-proteases are EDTA and EGTA.

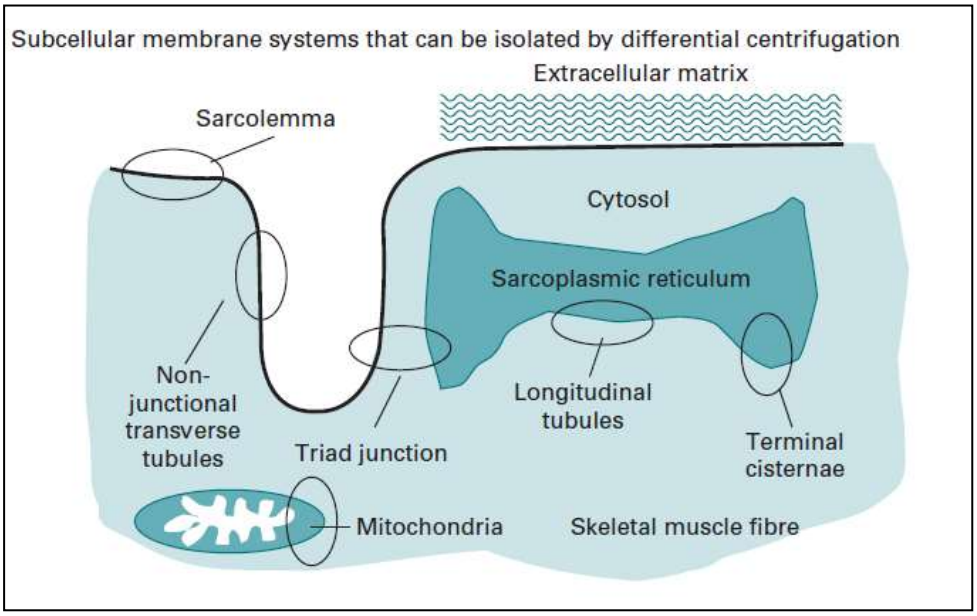


Figure: Scheme of the fractionation of skeletal muscle homogenate into various subcellular fractions. Shown is a diagrammatic presentation of the subcellular membrane system from skeletal muscle fibres.

Preparative vs Analytical Centrifugation:

Centrifugation is a separation method in which the application of the centrifugal force sediments or fractionates a heterogeneous mixture. Microcentrifugation, high-velocity centrifugation, and ultracentrifugation are the three types of centrifugation based on the volume and speed used in the process. Among other centrifugation methods, ultracentrifugation uses the maximum angular velocity. The rotating speed of ultracentrifugation is as high as 1 000 000 g. Thus, ultracentrifugation is used to isolate small particles such as ribosomes, proteins, and viruses. Preparative and analytical centrifugation are the two types of ultracentrifugation methods. The main difference between preparative and analytical centrifugation is that preparative centrifugation is used in pelleting small materials such as membranes, organelles, viruses**,** DNA and RNA whereas analytical centrifugation is used to determine the mass and shape of macromolecules such as protein complexes and rate of sedimentation of molecules**.**

Probable Questions:

1. Describe basic principle of centrifugation technique.
2. What is preparative centrifugation? Explain.
3. What is density gradient centrifugation? Explain.
4. Describe practical applications of preparative centrifugation.
5. Compare preparative vs analytical centrifugation.

Suggested Readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7^{th} Edition.
2. Biophysical Chemistry by Upadhyay and Upadhyay.

Unit-IX

Separation of Cell Constituents: I) Chromatography: ion exchange; gel filtration and HPLC II). Electrophoresis-PAGE, SDS-PAGE (One and Two dimensional)

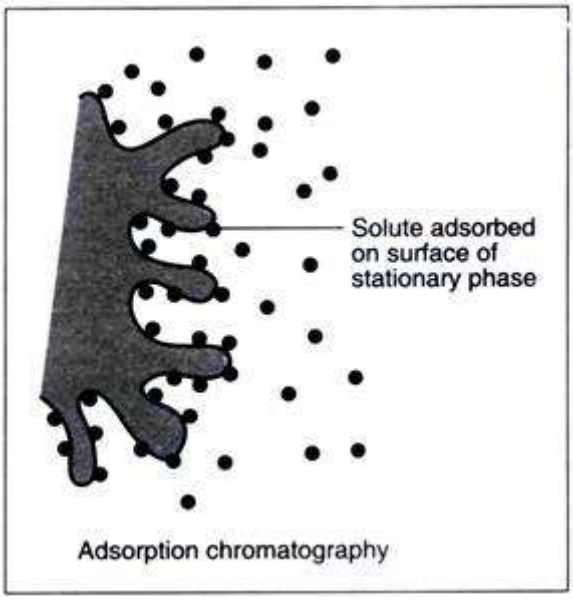
Objective: In this unit you will learn different separation techniques such as Chromatography and Electrophoresis.

I. Chromatgraphy:

Chromatography was originally introduced by Tswett in 1906, a Polish Botanist, for separation of different colour pigments present in the plant extract. Amino acids can also be separated from one another by partition chromatography.

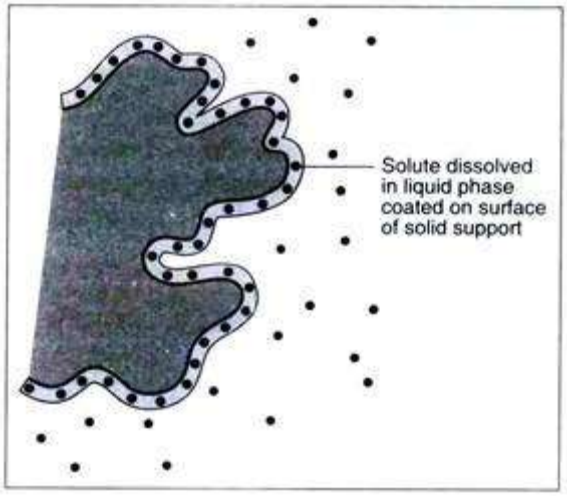
Adsorption Chromatography:

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phases accounts for the separation of different solutes.



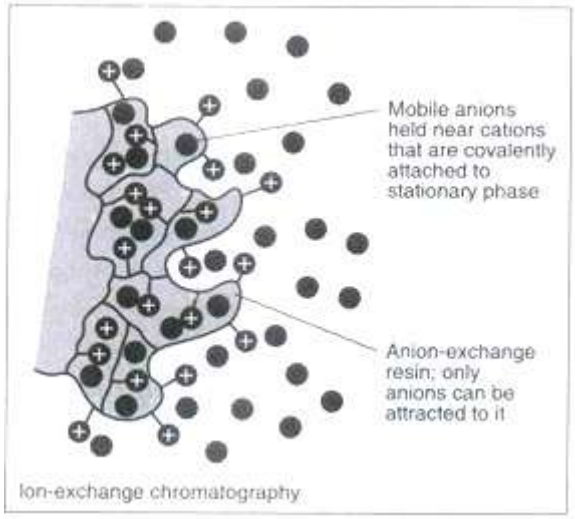
Partition Chromatography:

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.



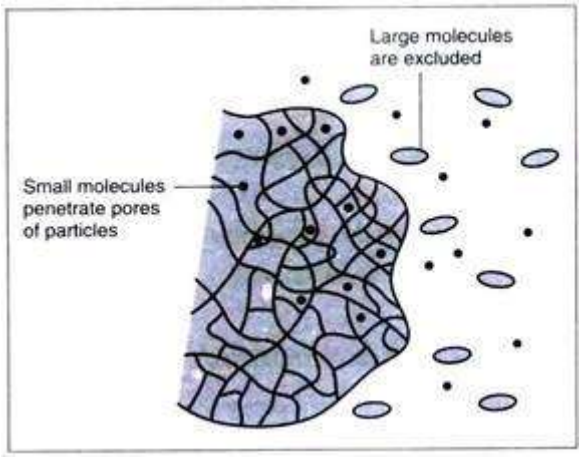
Ion Exchange Chromatography:

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.



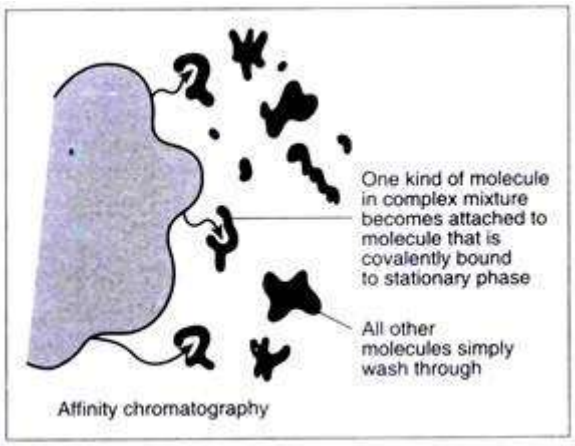
Molecular Exclusion Chromatography:

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.



Affinity Chromatography:

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of proteins are passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or pH.



Chromatography might be of different types, such as:

(a) Paper,
(b) Thin-layer,
(c) Column

The amino acids are separated between a stationary and a mobile phase. In paper chromatography (Fig. 2.2), a drop of amino acid mixture is placed on a filter paper and allowed to dry. The paper is then kept in contact with a suitable solvent which is allowed to flow over the dried drop slowly either by capillary action alone (ascending chromatography) or in combination with gravitational force (descending chromatography). As the solvent moves, it carries along with it the individual amino acids. Suitable tests are then applied to localise the individual amino acids which have been found to be carried away to a characteristic distance from the original place of application. The ratio of the distance travelled by the compound to the distance covered by the solvent on paper is called R_F value of the compound.

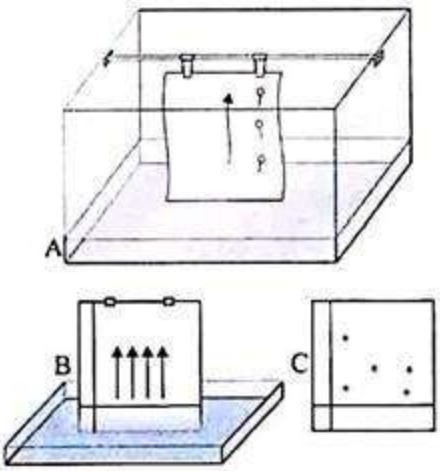


Fig. 2.2 Separation of amino acid mixture by paper chromatography. (A) placed in a suitable solvent the substances migrate upwards. (B & C), separation achieved by turning the paper 90° and by using a different solvent

To have a clearer separation, an improved method, the two-dimensional chromatography (Fig. 2.3) has been developed. Here after suitable chromatographic procedure, the paper is allowed to re-chromatograph at a right angle to the first one.

In thin-layer chromatography (TLC), a suitable adsorbent like alumina, cellulose powder, etc., is spread on glass plates which are then used as 'paper in chromatographic separation. This technique has some special advantages in separation.

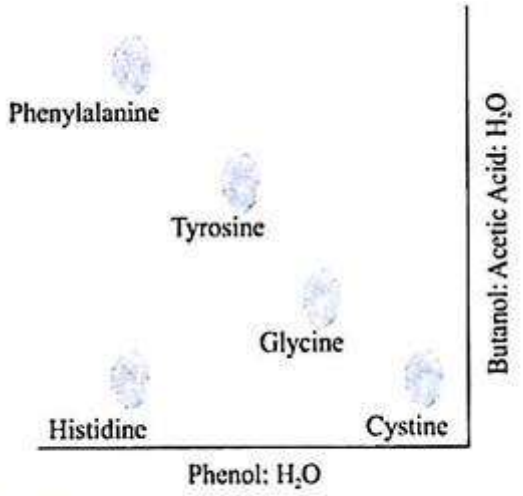


Fig. 2.3 Two-dimensional paper chromatography of some amino acids.

In column chromatography or ion-exchange chromatography, various solid adsorbents like starch, cellulose or an ion- pig. 2.4 exchange resins are placed as columns in glass tubes (Fig. 2.4). Different amino acids (or other substances) are adsorbed on the column are then eluted with suitable solvents.

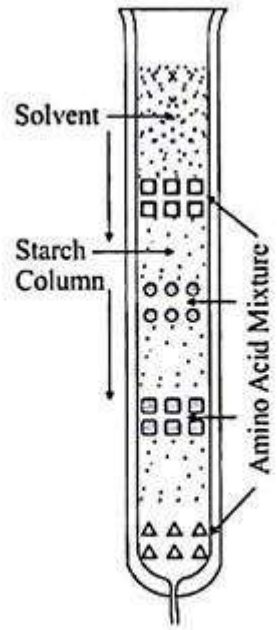


Fig. 2.4 Column chromatographic separation of amino acid mixture

A. Partition Chromatography:

This term covers liquid-liquid partition chromatography (Paper chromatography and thin layer chromatography). Gel chromatography, Gas liquid chromatography.

Paper Chromatography of Amino Acid Mixture:

Introduction:

In biology and chemistry, it is most often necessary to separate components of a mixture which are very similar and are difficult to separate by chemical or physical methods. Chromatography and electrophoresis arc two powerful modern methods utilised for such purpose.

Tswett (1906) the Russian biologist, first appreciated the possibilities of chromatography and he put the term "Chromatography". Consden. Gorden, and Martin (1944) described paper chromatography in which separations were done mainly by partition.

Principle:

The separation of components of a mixture by a chromatographic system depends on multiple containing the mixture of compounds is put on a strip of filter paper and allowed to dry. A partition process. Small differences in partitioning of each component of a mixture are multiplied many fold. The greater such differences the greater is the ease of separation.

A small drop of solution suitable solvent (mixture of two solvents) is allowed to flow along the filter paper over this spot. The substances in the initial spot are extracted by the flowing solvent and carried forward along the filter paper to a distance which appears related to their partition coefficient between the free and bound solvent phases of the filter paper. After the solvent has run for a suitable distance along the paper, the paper is removed, dried and subjected to suitable

tests to locate the various compounds. R_f value is defined as the ratio of the distance travelled by the component to the distance covered by the solvent. R_f value depends on the nature of the solvent, the temperature, and the presence of other substances.

Procedure:

Whatmann filter paper is cut into 35×15 cm sheet.

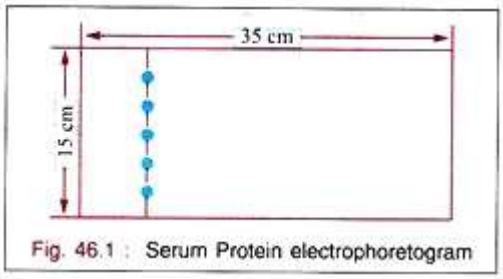


Fig. 46.1 : Serum Protein electrophoretogram

A pencil line is drawn about 3 cm above the shorter edge of the paper and 5 points are marked at equal spacing leaving 2.5 cm from the two edges. On the middle point, a mixture of four amino acids is applied with the help of a fine capillary. It is dried with the help of hot air blown by a hair dryer.

Again, another small quantity of the mixture is spotted at the same place and dried. This process is repeated 2 to 3 times more. On the other points, the individual amino acids of the mixture are similarly applied. The positions of these amino acids are marked with a pencil.

The solvent for developing the chromatogram is a mixture of n-butanol: acetic acid: water (12:3: 5) respectively. This mixture is freshly prepared. If a chromatographic tank is not available, specimen jar (about 40 x 15 cm) with fitting lid can very well serve the purpose.

In such ajar, put about 100 to 150 ml of the solvent mixture and replace the lid so that the lid is airtight. In case of doubts, apply Vaseline to the lid to avoid leakage. In an hour, the inside atmosphere will be saturated with the solvent vapour.

Now fold the paper in which sample has been applied in the shape of a cylinder and tie the opposing ends of the paper with staples or thread. Open the lid and place this folded paper in upright position in the jar, the pencil line lower most and about a centimeter above the solvent. Replace the lid. The paper should stand absolutely vertically. Leave the chromatogram to develop for 10 to 15 hours or earlier if the solvent has ascended quite near the upper margin of the paper.

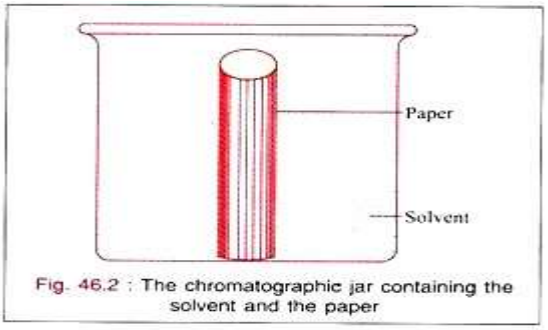


Fig. 46.2 : The chromatographic jar containing the solvent and the paper

Take out the paper at the desired time, cut the stitches and let it dry completely in the air. After the paper has dried thoroughly, the location reagent (0.2 per cent ninhydrin in acetone) is sprayed uniformly on the paper with the help of an all glass sprayer.
The paper is then allowed to dry first in the air and then in a hot air oven at 105°C for 3 minutes. Purple coloured amino acid spots are seen on the chromatogram. Identify the amino acids in the mixture with the help of spots produced by known amino acids.

The R_f values of the amino acids can be calculated as:
R_f = Distance travelled by particular amino acid/Distance covered by the solvent from the point of origin

Reagents:
a. N-butanol (Chromatographic grade).
b. Glacial acetic acid.
c. Solvent mixture of butanol, acetic acid, and distilled water in the proportion of (12 : 3 : 5)
d. Ninhydrin : 0.2 per cent solution of ninhydrin in acetone. It is prepared just before use.

B. Thin Layer Chromatography (TLC):
Introduction:
In recent years, thin layer chromatography has been developed. This technique consists of a thin layer of absorbents (silica gel, alumina. Kiselguhr or cellulose) on a glass plate or plastic sheet. Since absorbent is used, it is also termed as absorption chromatography.
This technique also provides superior results than that of paper chromatography. The spots are more compact with better resolution and the run is comparatively of shorter duration. Therefore, quicker run is possible.

Preparation of Plates:
Chromatographic plates (20 x 20 cm) of 200 μ thickness are prepared by using a suspension of 30 grams of silica gel G in 63 ml of 0.1 M Na_2CO_3 solution by shaking vigorously for 90 seconds. Only these plates are used which appear to be uniform in both transmitted and reflected light. These plates are activated at 110°C for 30 minutes immediately prior to use.

Table 46.1 : Electrophoretic pattern changes in some conditions, '—' mark indicate no change

Disease	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin
Normal (gm/100ml)	3.5 to 5.5	0.1 to 0.4	0.4 to 0.8	0.5 to 1.0	0.7 to 1.5
Infancy	—	high	high	—	—
Pregnancy	low	high	high	high	—
Acute infection	—	high	high	—	—
Chronic infection	low	high	high	—	high
Cirrhosis	low	high	—	—	high
Nephrotic syndrome	low	high	very high	—	low
Myelomatosis	—	—	—	—	high
Hypogammaglobinemia	—	—	—	—	very low

Separate band occassionally seen in α and β position

Procedure:

Samples (5-100 (JL) are applied as a spot of less than 5 mm diameter on the lower right corner of the plates under a stream of warm air. Plates are first developed in a standard Brinkmann developing chamber previously saturated with the vapour of the solvent mixture with chloroform: methanol: acetic acid: water (250 : 74 : 19 : 3 ; V/V).

When the solvent front migrates about 15 cm, plates are dried in air for 15 minutes and develop in the second dimension (90° rotation clockwise) with chloroform: methanol: 7 M. ammonium hydroxide (230 : 90 : 15 ; V/V). The solvent front is again allowed to move about 15 cm.

Developed plates are then dried in air for 5 minutes and exposed to iodine vapour in a sealed chamber for 30 to 60 seconds. The pale yellow areas are quickly outlined using a dental probe and the plates are exposed to air until the iodine has evaporated from the spots. When a permanent record of developed plates is desired, plates are sprayed lightly with 10 N. H_2SO_4 and then heated at 110°G for 15 minutes.

The silica gel in each spot is scraped with the aid of a sharp edged polyethylene blade on paper. The weighing papers are then transferred to a 12 ml conical centrifuge tube and eluted by different solvents for estimation by photoelectric colorimeter.

Discussion:

The constituents of the mixture of amino acids, and the constituents of neutral lipids and phospholipids are separated and estimated in a short time.

C. Gel Chromatography:

(i) This type of Chromatography uses a porous gel. The dry gel particles are first allowed to take up the chosen solvent. This is accompanied by swelling; the liquid taken up constitutes the stationary phase.

(ii) These swollen particles are when made into a column with the same solvent, the spaces between them are filled by solvents. The mobile phase is the “void volume” (Vo); the gel particles are sponge like and the channels within them are of similar diameter (pore size) for a particular grade of gel.

(iii) Molecules can enter the stationary phase only within the gel if their diameter is less than this pore-size. Thus small molecules have the whole fluid volume (bed volume), but molecules above this limit are confined to the void volume and are rapidly washed through the column as mobile phase.

(a) This type of separation is usually performed using the pressure of a small head of the liquid phase to force this through the column. By per-fusing the mobile phase at much higher pressures the separation can be carried out quickly with high resolution.

(b) Dense column packing gives high resolution and high pressure metering systems maintain extremely reproducible solvent flows.

(c) This equipment consists of a column, a solvent delivery system, a sample injector and a detection. The column is of stainless steel of 10-50 cm long and 2-5 mm internal diameter. The

solvent delivery is effected by solvent pumps which generate pressure at several thousand Psi and direct a pulse-free delivery of the solvent.

Constant pressure and constant volume pumps are used for the purpose. The sample injection is done with a syringe. The detectors may be ultraviolet with fixed wavelength Hg lamp 254 nm, refractive index detectors of deflection type, fluorescence detectors.

(d) This method is highly applicable for the separation of carbohydrates, proteins, peptides, amino acids, vitamins, steroids, neuropeptides, hormones and drugs. Separations are achieved very quickly. Hence this method is indispensable in laboratories where advance research and sophisticated analyses are performed.

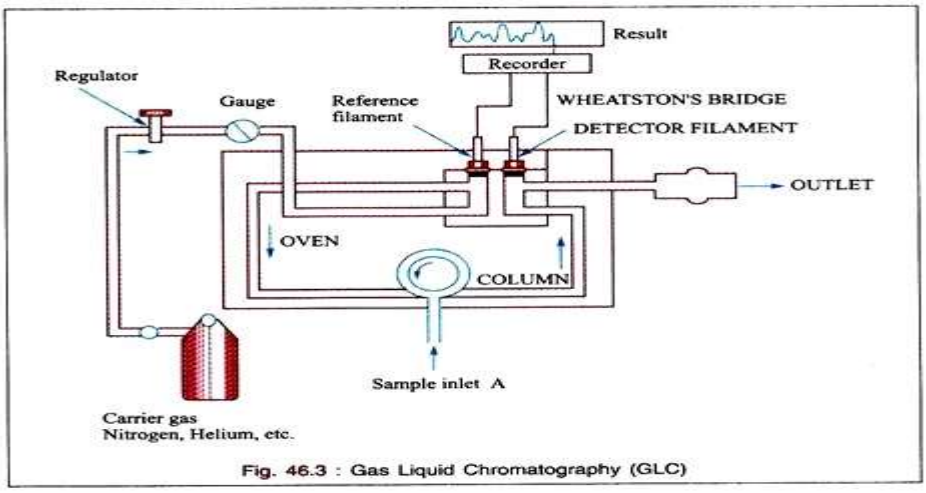


Fig. 46.3 : Gas Liquid Chromatography (GLC)

D. Gas Liquid Chromatography (GLC):

(i) The substances to be separated are carried as vapours in an inert gas like nitrogen, argon or helium over liquids when there is partitioning of the substances between the gas and the liquid. The liquids used are silicone, oils, lubricating greases, etc. held in inert solids like di-aomaceous earth or ground firebrick.

(ii) Glass or metal tube 1-2 metres long and of 0.2 – 2 cm diameter can be used.

(iii) If fatty acids are to be separated, they are first converted to methyl esters which are easily evaporated. The vapours are swept constantly by nitrogen through the tube containing the liquid phase at a temperature of 170-225°C, so that the vapours of the esters may remain as vapours. Separation takes places owing to the partitioning of the esters between the gas and the liquid. The ingredients can be identified by physical, chemical means or flame ionisation.

E. Ion-Exchange Chromatography:

(i) Ion-exchange resins are nothing but cross-linked polymers. The polymers must have negligible solubility but be porous enough for the ions to diffuse freely through it.

(ii) Ion-exchange resins are of cation and anion exchangers. Strong cation-exchange resins contain sulphuric acid groups ($-SO_3^-$), weak ones carboxylic acid groups ($-COO^-$), whereas strong anion-exchange resins have -N (R_1 R_2 R_3) and weak ones —N(R_1 R_2).

(iii) The most important resins are polystyrene resins formed by condensation of styrene (vinyl benzene) and divinyl benzene. Acidic or basic groups are introduced before or after polymerizing.
(iv) Strong alkaline cellulose treated with chloroacetic acid introduces the carboxy methyl group to give the weak cation-exchange resin carboxymethyl-cellulose (CM-cellulose) while condensation with 2-chlorotriethylamine gives the weak anion-exchange diethylaminoethyl-cellulose (DEAE-cellulose). Cellulose ion-ex-change materials are specially suitable for protein separations.
(v) The resins can be looked on as insoluble acids or bases which form insoluble salts shown below.
$H^+ – Resin^- + Na^+ \rightarrow Na^+ – Resin^- + H^+$ (Cation-exchanger)
$esin^+ – OH^- + CI^- \rightarrow Resin^+ – CI^- + OH^-$ (Anion-exchanger)
The more strongly acidic the ion-change resin, the greater is the ionisation of the acidic group and the lower the pH at which it will exchange.

(vi) Ion-exchange resins have been widely used for the separation of amino acids and peptides. These have also been used to separate organic weak cations or anions from inorganic salts – ion-exchange resulting.
(vii) Mixed beds of anion and cation exchange resins have the property of replacing cations and anions of any salt in water by equivalent amounts of H^+ and OH^- respectively. This process is used in the preparation of "deionised" water in the laboratory. Non-ionic contaminants are not removed.

F. Gel Filtration Chromatography:
Gel Filtration Chromatography or Size exclusion chromatography (SEC) is a chromatographic method in which particles are separated based on their size, or in more technical terms, their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. When an aqueous solution is used to transport the sample through the column, the technique is known as gel filtration chromatography.
The name gel permeation chromatography is used when an organic solvent is used as a mobile phase. The main application of gel filtration chromatography is the fractionation of proteins and other water-soluble polymers, while gel permeation chromatography is used to analyse the molecular weight distribution of organic-soluble polymers. Either technique should not be confused with gel electrophoresis, where an electric field is used to "pull" or "push" molecules through the gel depending on their electrical charges.
SEC is a widely used technique for the purification and analysis of synthetic and biological polymers, such as proteins, polysaccharides and nucleic acids. Biologists and biochemists typically use a gel medium—usually polyacrylamide, dextran or agarose—and filter under low pressure. Polymer chemists typically use either a silica or cross-linked polystyrene medium under a higher pressure. These media are known as the stationary phase.

The advantage of this method is that the various solutions can be applied without interfering with the filtration process, while preserving the biological activity of the particles to be separated. The technique is generally combined with others that further separate molecules by other characteristics, such as acidity, basicity, charge, and affinity for certain compounds.

The underlying principle of SEC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This result in the separation of a solution of particles based on size, provided that all the particles are loaded simultaneously or near simultaneously, particles of the same size should elute together.

This is usually achieved with an apparatus called a column, which consists of a hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes. These pores may be depressions on the surface or channels through the bead. As the solution travels down the column some particles enter into the pores. Larger particles cannot enter into as many pores. The larger the particles, the less overall volume to traverse over the length of the column, and the faster the elution.

The filtered solution that is collected at the end is known as the eluent. The void volume consists of any particles too large to enter the medium, and the solvent volume is known as the column volume. In real life situations, particles in solution do not have a constant, fixed size, resulting in the probability that a particle which would otherwise be hampered by a pore may pass right by it. Also, the stationary phase particles are not ideally defined; both particles and pores may vary in size.

Elution curves, therefore, resemble Gaussian distributions. The stationary phase may also interact in undesirable ways with a particle and influence retention times, though great care is taken by column manufacturers to use stationary phases which are inert and minimize this issue.

Like other forms of chromatography, increasing the column length will tighten the resolution, and increasing the column diameter increases the capacity of the column. Proper column packing is important to maximize resolution: an over packed column can collapse the pores in the beads, resulting in a loss of resolution. An under packed column can reduce the relative surface area of the stationary phase accessible to smaller species, resulting in those species spending less time trapped in pores.

In simple manual columns the eluent is collected in constant volumes, known as fractions. The more similar the particles are in size, the more likely they will be in the same fraction and not detected separately. More advanced columns overcome this problem by constantly monitoring the eluent. The collected fractions are often examined by spectroscopic techniques to determine the concentration of the particles eluted. Three common spectroscopy detection techniques are refractive index (RI), evaporative light scattering (ELS), and ultraviolet (UV). When eluting spectroscopically similar species (such as during biological purification) other techniques may be necessary to identify the contents of each fraction.

The elution volume decreases roughly linearly with the logarithm of the molecular hydrodynamic volume (often assumed to be proportional to molecular weight). Columns are

often calibrated using 4-5 standard samples (e.g., folded proteins of known molecular weight) to determine the void volume and the slope of the logarithmic dependence. This calibration may need to be repeated under different solution conditions.

Applications:

1. Proteomics:

SEC is generally considered a low resolution chromatography as it does not discern similar species very well, and is, therefore, often reserved for the final "polishing" step of a purification. The technique can determine the quaternary structure of purified proteins which have slow exchange times, since it can be carried out under native solution conditions, preserving macromolecular interactions.

SEC can also assay protein tertiary structure as it measures the hydrodynamic volume (not molecular weight), allowing folded and unfolded versions of the same protein to be distinguished. For example, the apparent hydrodynamic radius of a typical protein domain might be 14a and 36A for the folded and unfolded forms respectively.

SEC allows the separation of these two forms as the folded form will elute much later due to its smaller size. Alternatively, folded and unfolded versions of the same metalloproteinase can be separated according to their different isoelectric points by using quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE).

2. Polymer synthesis:

SEC can be used as a measure of both the size and the polydispersity of a synthesised polymer; that one is able to find distribution of sizes of polymer molecules. If standards of a known size are run previously, then a calibration curve can be created to determine the sizes of polymer molecules of interest. Alternatively, techniques such as light scattering and/or viscometry can be used online with SEC to yield absolute molecular weights that do not rely on calibration with standards of known molecular weight. Due to the difference in size of two polymers with identical molecular weights, the absolute determination methods are generally more desirable. A typical SEC system can quickly (in about half an hour) give polymer chemists information on the size and polydispersity of the sample.

G. High Performance Liquid Chromatography:

High-performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry. It is also sometimes referred to as high-pressure liquid chromatography. HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analysed (analyte) and the chromatography column.

In isocratic HPLC, the analyte is forced through a column of the stationary phase (usually a tube packed with small round particles with a certain surface chemistry) by pumping a liquid

(mobile phase) at high pressure through the column. The sample to be analysed is introduced in a small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column.

The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram.

Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as Trifluoroacetic acid which acts as an ion pairing agent. A further refinement to HPLC has been to vary the mobile phase composition during the analysis; this is known as gradient elution. A normal gradient for reverse phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes, depending on how hydrophobic the analyte is.

The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/methanol gradient, the more hydrophobic components will elute (come off the column) under conditions of relatively high methanol; whereas the more hydrophilic compounds will elute under conditions of relatively low methanol.

The choice of solvents, additives and gradient, depends on the nature of the stationary phase and the analyte. Often a series of tests are performed on the analyte and a number of generic runs may be processed in order to find the optimum HPLC method for the analyte — the method which gives the best separation of peaks.

Types of HPLC:

1. Normal phase chromatography:

Normal phase HPLC (NP-HPLC) was the first kind of HPLC chemistry used, and separates analytes based on polarity. This method uses a polar stationary phase and a non-polar mobile phase, and is used when the analyte of interest is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase.

Adsorption strengths increase with increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on the functional groups in the analyte molecule, but also on steric factors, and structural isomers are often resolved from one another. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes while more hydrophobic solvents tend to increase retention times. Particularly polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface. This is

somewhat particular to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).
NP-HPLC had fallen out of favour in the 1970s with the development of reversed-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently it has become useful again with the development of HILIC bonded phases which utilize a partition mechanism which provides reproducibility.

2. Reversed phase chromatography:

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and a moderately polar mobile phase. One common stationary phase is a silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is, therefore, longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily.
Retention time is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. Reversed phase chromatography is so commonly used that it is not uncommon for it to be incorrectly referred to as "HPLC" without further specification.
RP-HPLC operates on the principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyte, and the non-polar stationary phase. The driving force in the binding of the analyte to the stationary phase is the decrease in the area of the non-polar segment of the analyte molecule exposed to the solvent.
This hydrophobic effect is dominated by the decrease in free energy from entropy associated with the minimization of the ordered molecule-polar solvent interface. The hydrophobic effect is decreased by adding more non-polar solvent into the mobile phase. This shifts the partition coefficient such that the analyte spends some portion of time moving down the column in the mobile phase, eventually eluting from the column.
The characteristics of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a longer alkyl chain length results in a longer retention time because it increases the molecule's hydrophobicity.
Very large molecules, however, can result in incomplete interaction between the large analyte surface and the alkyl chain. Retention time increases with hydrophobic surface area which is roughly inversely proportional to solute size. Branched chain compounds elute more rapidly than their corresponding isomers because the overall surface area is decreased.
Apart from mobile phase hydrophobicity, other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a linear increase in the surface tension of aqueous solutions, and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tends to increase the retention time.
Another important component is pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the

pH. An organic acid such as formic acid or most commonly trifluoro-acetic acid is often added to the mobile phase.

These serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralize charge on the analyte. The effect varies depending on use but generally improves the chromatography.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reverse phase columns consist of alkyl derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica backbone. They can be used with aqueous acid but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment.

The metal content of HPLC columns must be kept low if the best possible ability to separate substances is to be retained. A good test for the metal content of a column is to inject a sample which is a mixture of 2, 2′- and 4, 4′- bipyridine. Because the 2,2′- bipyridine can chelate the metal it is normal that when a metal ion is present on the surface of the silica the shape of the peak for the 2,2′-bipyridine will be distorted, tailing will be seen on this distorted peak.

Parameters:

There are different parameters upon which the separation by HPLC relies. Some of the important parameters are discussed below:

1. Internal diameter:

The internal diameter (ID) of an HPLC column is a critical aspect that determines quantity of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

i. Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.

ii. Analytical scale columns (4.6 mm) have been the most common type of columns, though smaller columns are rapidly gaining popularity. They are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.

iii. Narrow-bore columns (1-2 mm) are used for applications when more sensitivity is desired either with special UV-Vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry

iv. Capillary columns (under 0.3 mm) which are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

2. Particle size:

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 μm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

This means that changing to particles that are half as big in the same size of column will double the performance, but increase the required pressure by a factor of four. Larger particles are more often used in non-HPLC applications such as solid-phase extraction.

3. Pore size:

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but not easily leave once inside.

4. Pump pressure:

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 6000 lbf/in^2 (~40 MPa, or about 400 atmospheres). Modern HPLC systems have been improved to work at much higher pressures, and therefore, be able to use much smaller particle sizes in the columns (< 2 micrometres).

These "Ultra High Performance Liquid Chromatography" systems or UHPLCs can work at up to 15,000 lbf/in^2 (-100 MPa or about 1000 atmospheres). Note that the term "UPLC", sometimes found instead is a trademark of Waters Corporation and not the name for the technique in general.

II. Electrophoresis:

The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any pH, exist in solution as electrically charged species either as cations (+) or anions (-).

Under the influence of an electric field charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

In order to understand fully how charged species separate, it is necessary to look at some simple equations related to electrophoresis. When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient, E, which is applied voltage, V, divided by the distance, d, between the electrodes. When this potential gradient E is applied, the force on a molecule bearing a charge of q coulombs is Eq newton's.

It is this force that derives a charged molecule towards an electrode. However, there is also a factional resistance that retards the movement of this charged molecule. This frictional force is a measure of the hydrodynamic size of the molecule, the shape of the molecule, the pore size of the medium in which electrophoresis is taking place and the viscosity of the buffer.

The velocity, v, of a charged molecule in electrical field is, therefore, given by the equation: $v = Eq/f$, where f is the frictional coefficient.

More commonly the term electrophoretic mobility (μ) of an ion is used, which is the ratio of the velocity of the ion to field strength (v/E). When a potential difference is applied, therefore, molecules with different overall charges will begin to separate owing to their different electrophoretic nobilities.
Even molecules of similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces. As will be seen below, some forms of electrophoresis rely almost totally on the different charges on the molecules to effect separation, whilst other methods exploit differences in molecular size and, therefore, encourage frictional effects to bring about separation.

In short, electrophoretic mobility can be given as:
Mobility = (voltage) (charge)/ (frictional coefficient)

Provided the electric field is removed before the molecules in the sample reach the electrodes, the components will have been separated according to their electrophoretic mobility. Electrophoresis is thus an incomplete form of electrolysis. The separated samples are then located by staining with an appropriate dye or by autoradiography if the sample is radiolabelled. The current in the solution between the electrodes is conducted mainly by the buffer ions, a small proportion being conducted by the sample ions. Ohm's law expresses the relationship between current (I), voltage (V) and resistance (R):

$$V/I = R.$$

It, therefore, appears that it is possible to accelerate an electrophoretic separation by voltage, which would result in a corresponding increase in the current flowing. The distance migrated by the ions will be proportional to both current and time. However, this would ignore one of the major problems for most common forms of electrophoresis, namely the generation of the heat.

During electrophoresis, the power (W, watts) generated in the supporting medium is given by: $W = I^2R$.

Most of this power generated is dissipated as heat. Heating of the electrophoretic medium has the following effects:
i. An increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples.
ii. The formation of convection currents, which leads to mixing of the separated samples.
iii. Thermal instability of the samples that are rather sensitive to heat. This may include denaturation of the proteins (e.g., the loss of enzyme activity).

iv. A decrease of buffer viscosity, and hence a reduction in the resistance of the medium.

If a constant voltage is applied, the current increases during electrophoresis owing to the decrease in the resistance and the rise in current increases the heat output still further. For this reason, workers often use a stabilized power supply, which provides constant power and thus eliminates fluctuations in heating.

Constant heat generation is, however, a problem. One may think that electrophoresis may be run at a very low power (low current) to overcome any heating problem, but this can lead to poor separation as a result of the increased amount of the diffusion resulting from a long separation times. Compromise conditions, therefore, have to be found with reasonable power settings, to give acceptable separation times, and an appropriate cooling system, to remove liberated heat.

While such systems work fairly well, the effects of heating are not always totally eliminated. For example, for electrophoresis carried out in cylindrical tubes or in slab gels, although heat is generated uniformly through the medium, heat is removed only from the edges, resulting in a temperature gradient within the gel, the temperature at the centre of the gel being higher than that at the edges.

Since the warmer fluid at the centre is less viscous, electrophoretic mobility are, therefore, greater in the central region (electrophoretic mobilities increase by about 2% for each 1°C rise in the temperature), and electrophoretic zones develop a bowled shape, with the zone centre migrating faster than the edges.

A final factor that can effect electrophoresis separation is the phenomenon of electroendoosmosis (also known as electro-osmotic flow), which is due to the presence of charged groups on the surface of the support medium. For example, paper has some carboxyl groups present, agarose (depending on the purity grade) contains sulphate group and the surface of the glass walls used in capillary electrophoresis contains silanol (Si-OH) groups.

Mechanism of electroendoosmosis has been explained in details in the section covering capillary electrophoresis, although the principle is the same for any support medium that has charged groups on it. In short in a fused silica capillary tube, above a pH value of about 3, silanol groups on the silica capillary walls will ionise, generating negatively charged sites. It is these charges that generate electroendoosmosis.

The ionized silanol groups create an electrical double layer, or region of charge separation, at the capillary wall/electrolyte interface. When a voltage is applied, cations in the electrolyte near the capillary wall migrate towards the cathode, pulling electrolyte solution with them. This creates a net electrosmotic flow towards the cathode.

However, the introduction of the use of gels as a support medium led to a rapid improvement in methods for analyzing macromolecules. The earliest gel system to be used was the starch gel and, although this has some uses, the vast majority of electrophoretic techniques used nowadays involve either agarose gel or polyacrylamide gel.

Agarose Gel Electrophoresis:

Agarose is a linear polysaccharide (average relative molecular mass about 12000) made up of the basic repeat unit agarobiose, which comprises alternating units of the galactose and 3, 6-anhydrogalactose (Fig. 8.4).

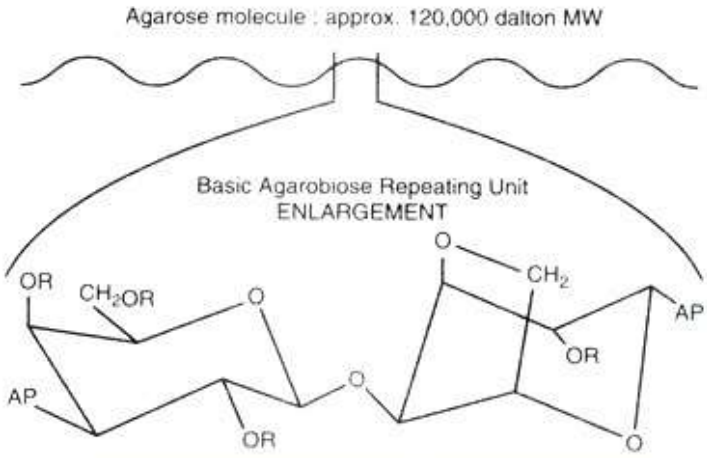


Fig. 8.4: Structure of agarose

Agarose is one of the components of agar that is mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at a concentration between 1% and 3%. Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution is formed. This is poured and allowed to cool to room temperature to form a rigid gel. The gelling properties are attributed to both inter- and intermolecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anti-conventional properties. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentration and smaller pore sizes are formed from higher concentrations.

Although essentially free from charge, substitution of the alternating sugar residues with carboxyl, methoxyl, pyruvate and specially sulphate group occur to varying degrees. This substitution can result in electroendoosmosis during electrophoresis and ionic interactions between the gel and sample in all uses, both unwanted effects. Agarose is, therefore, sold in very different purity grades, based on sulphate concentration — the lower the sulphate content the higher the purity.

Agarose gels are used for the electrophoresis of both proteins and nucleic acids. For proteins, the pore sizes of a 1% agarose gel are large relative to the sizes of proteins. Agarose gels are therefore used in techniques such as Immunoelectrophoresis or flat bed isoelectric focusing, where the proteins are required to move unhindered in the gel matrix according to their native charge.

Such large pore gels are also used to separate much larger molecules such as DNA or RNA, because the pore sizes in the gel are still large enough for DNA or RNA molecule to pass through the gel. Now, however, the pore size and molecule size are more comparable and frictional effects begin to play a role in the separation of these molecules.

A further advantage of using agarose is the availability of low melting temperature agarose (62-65°C). As the name suggests, these gels can be re-liquefied by heating to 65°C and thus, for example, DNA samples separated in a gel can be cut out of the gel, returned to solution and recovered.

Table 8.1: Range for fractionation of different compounds in different agarose percentages

Agarose Concentration	Protein Fractionation Range* (kDa)	Polysaccharide Fractionation* (kDa)	Nucleic Acid Exclusion Limit (bp)
1.0%	1,000 to 150,000	1,000 to 150,000	(> 3,000)
2.0%	80 to 40,000	90 to 20,000	1,340
4.0%	50 to 15,000	40 to 5,000	860
6.0%	10 to 5,000	10 to 1,000	180

Owing to the poor elasticity of agarose gels and the consequent problems of removing them from small tubes, the gel rod system is sometimes used, since acrylamide gel is not used. Horizontal slab gels are invariably used for isoelectric focusing or immunoelectrphoresis in agarose. Horizontal gels are also used routinely for DNA and RNA gels, although vertical systems have been used by some workers.

Table 8.2: Approximate gel concentrations for separation of DNA linear fragments of various sizes

Gel Concentration	Separation Range (bp)
agarose - 0.3%	60,000 - 5,000
agarose - 0.7%	20,000 - 800
agarose - 0.9%	7,000 - 500
agarose - 1.2%	6,000 - 400
agarose - 1.5%	4,000 - 200
agarose - 2.0%	3,000 - 100
agarose - 4.0%	500 - 10
acrylamide - 4%	1,000 - 800
acrylamide - 10%	500 - 25
acrylamide - 20%	50 - 1

Agarose Gel Electrophoresis of Nucleic Acids:

Nucleic acids are polymers composed of individual nucleotide units. The units are connected via phosphate diester linkages of the backbone sugars. The net effect of these linkages is to give the polymers a net negative charge. From the earliest days of electrophoresis it has been axiomatic that molecules carrying an electrical charge will migrate in an electrical field in a predictable manner.

When subjected to an electrical field, a molecule carrying a net negative charge will migrate toward the positive pole and a molecule with a net positive charge will migrate toward the negative pole. In a semi-solid matrix like agarose, the equation describing mobility can be re-interpreted, at least heuristically, by defining gas gel density or concentration and r as the length of the molecule.

Thus, when they are placed in the semi-solid matrix of a gel, nucleic acids will migrate toward the positive pole in a predictable and reproducible manner that can be described as a negative exponential function of length. That is to say, shorter molecules will migrate faster and longer molecules will migrate slower. Indeed, in the case of a nucleic acid in a gel in an electrical field, every other element of the migration expression is a constant and mobility is completely determined by molecular length.

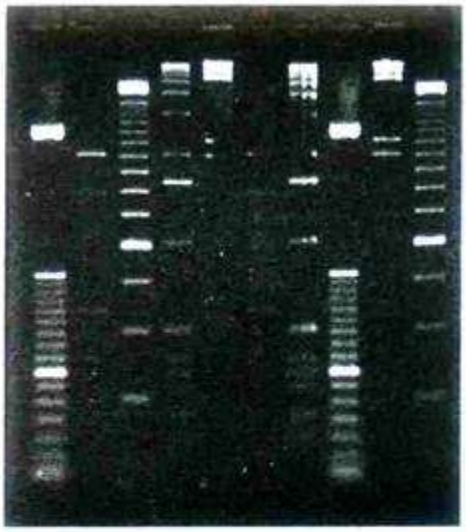


Fig. 8.6: DNA separated on 0.8% agarose gel (*Courtesy*: Dr D Dash, Deptt. of Biochemistry, IMS, BHU)

As a matter of practice, it is difficult to accurately resolve double-stranded nucleic acids smaller than about 100 bases in an agarose gel because the sieving properties of agarose are not fine enough. On the other end of the scale, molecules longer than about 25,000 bp but shorter than around 2,000,000 bp will all run at the same rate. This is called limiting mobility.

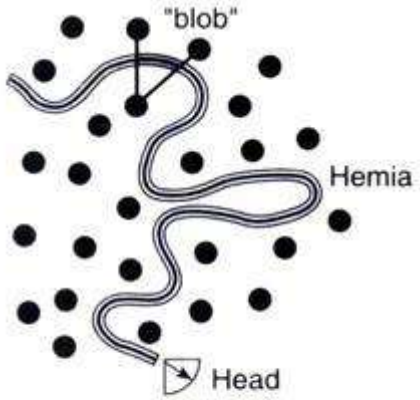


Fig. 8.7: Biased reptation

Nucleic acid molecules longer than 2,000,000 bp will not even enter an agarose gel. Thus, the effective size range for agarose gel electrophoresis of double stranded nucleic acids is between

100 bp and 25,000 bp. In this range the behaviour of the molecule is precise and predictable. This behaviour is shown in Fig. 8.8. As can be seen there is minimal separation of the larger fragments but resolution improves as the fragments get smaller.

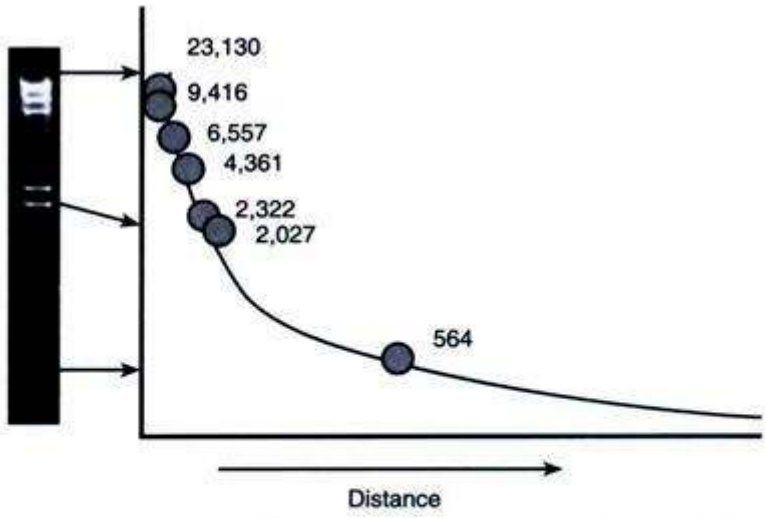


Fig. 8.8: Electrophoretic mobility of DNA restriction fragments in an agarose gel. The DNA is from bacteriophage lambda digested with the restriction enzyme *Hind III*. The graph shows migration distance in centimetres by size of the restriction fragment in base pairs.

While this phenomenon has been known for many years, what was not known was how the nucleic acid molecules actually moved in the gel matrix. In the 1980s a theory was put forward that nucleic acids migrated through the gel much the same way that a snake moves. That is, the leading edge moves forward and pulls the rest of the molecule with it.

In this model, as the molecule gets longer resistance being pulled along increases. This resistance is further increased by the interaction of the molecule with the gel matrix. The increase in resistance is non-linear. This model, called "biased reptation", is sufficient to explain all of the behaviour of a nucleic acid in a semi-solid matrix (Lerman et al, 1982; Lumpkin et al., 1985).

In 1989 a group at the University of Washington put this theory to the test. They filmed DNA molecules moving through an agarose gel. Their films showed both reptation and the nucleic acid/ gel matrix interactions (Smith et al., 1989).

In the mid-1980s a number of methods were developed to electrophoretically analyse nucleic acid molecules in the limiting mobility size range. The solution involved artificially introducing a size dependent mobility on nucleic acid molecules by altering the electrophoretic field. The first such alteration involved simply switching the polarity of the field in a regular pattern. Carle et al. (1986) showed that periodic reversals of polarity would induce the molecules to make U-turns in the gel.

Even at very large sizes, this turning would permit separation of molecules. In the length of time the molecules were reversed was about one-third the time they were oriented forward, for example, three seconds forward and one second back, molecules as large as 2,000,000 bp could be resolved in a standard agarose gel in a few hours. The first practical demonstration of this method, called Field Inversion Gel Electrophoresis (FIGE), was to completely resolve intact yeast chromosomes. Since then, a variety of methods, collectively termed pulsed-field gel electrophoresis, have been developed.

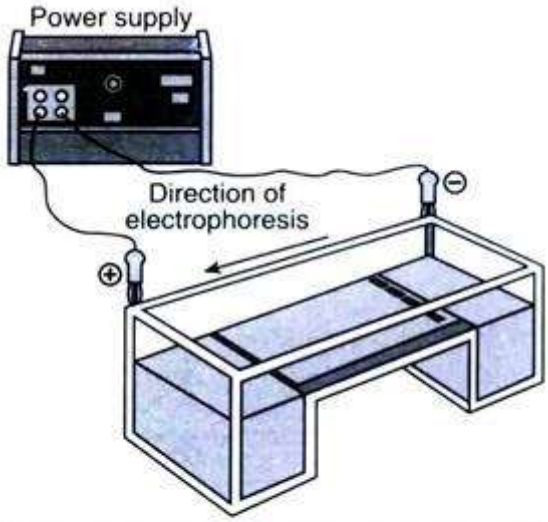


Fig. 8.9: A typical horizontal gel electrophoresis system

Many laboratories routinely use 0.8% gels, which are suitable for separating DNA molecules in the range 0.5- 10 kb. Since agarose gels separate DNA according to size, the M_r of a DNA fragment may be determined from its electrophoretic mobility by running a number of standard DNA markers of known M_r on the same gel. This is most conveniently achieved by running a sample of bacteriophage λ DNA (49 kb) that has been cleaved with a restriction enzyme such as EcoRI. Since the base sequence of λ DNA is known, and the cleavage sites for EcoRl are known, this generates fragments of accurately known size.

DNA gels are invariably run as horizontal, submarine or submerged gels; so named because such a gel is totally immersed in buffer. Agarose, dissolved in gel buffer by boiling, is poured onto a glass or plastic plate, surrounded by a wall of adhesive tape or a plastic frame to provide a gel about 3 mm in depth. Loading wells are formed by placing a plastic well-forming template or comb in the poured gel solution, and removing this comb once the gel is set.

The gel is placed in electrophoresis tank, covered with buffer, and samples loaded by directly injecting the sample into the wells. Samples are prepared by dissolving them in a buffer solution that contains sucrose, glycerol or Ficoll, which makes the solution dense and allows it to sink to the bottom of the well. A dye such as bromophenol blue is also included in the sample solvent; it makes it easier to see the sample that is being loaded and also acts as a marker of the electrophoresis front. No stacking gel is needed for the electrophoresis of DNA because the mobilities of DNA molecules are much greater in the well than in the gel, and therefore, all the molecules in the well pile up against the gel within a few minutes of the current being turned on, forming a tight band at the start of the run.

General purpose gels are approximately 25 cm long and 12 cm wide, and are run at a voltage gradient of about 1.5 V cm^{-1} overnight. A higher voltage would cause excessive heating. For rapid analyses that do not need extensive separation of DNA molecules, it is common to use mini-gels that are less than 10 cm long. In this way information can be obtained in 2-3 h.

Fig. 8.10: Horizontal gel electrophoresis system (*Courtesy:* Dr D Dash, Deptt. of Biochemistry, IMS, BHU)

Once the system has been run, the DNA in the gel needs to be stained and visualized. The reagent most widely used is the fluorescent dye ethidium bromide. The gel is rinsed gently in a solution of ethidium bromide (0.5 µg cm^{-1}) and then viewed under ultraviolet light (300 nm wavelength). Ethidium bromide is a cyclic planar molecule that binds between the stacked base-pairs of DNA (i.e., it intercalates).

The ethidium bromide concentration, therefore, builds up at the site of DNA bands and under ultraviolet light the DNA bands fluoresce orange-red. As little as 10 ng of DNA can be visualized as a 1 cm wide band. It should be noted that extensive viewing of DNA with ultraviolet light can result in damage of the DNA by nicking and base-pair dimerization.

This is of no consequence if the gel is only to be viewed, but obviously viewing of the gel should be kept to a minimum if the DNA is to be recovered. It is essential to protect one's eye by wearing goggles when ultraviolet light is used. If viewing of gel under ultraviolet is carried out for long periods, a plastic mask that covers the whole face should be used to avoid 'sunburn'.

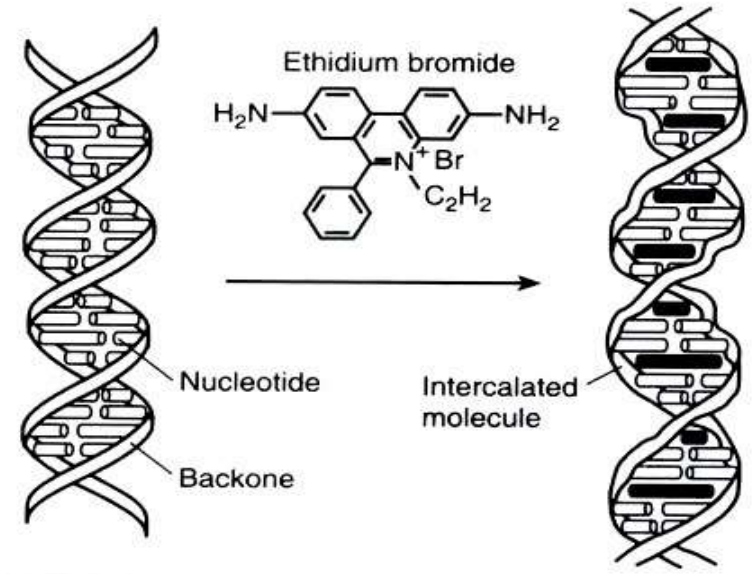


Fig. 8.11: Interaction of ethidium bromide with DNA molecule

DNA Sequencing Gels:

Although agarose gel electrophoresis of DNA is a 'workhorse' technique for the molecular biologists, a different form of electrophoresis has to be used when DNA sequences are to be determined. Whichever DNA sequencing method is used, the final analysis usually involves separating single-stranded DNA molecules shorter than about 1000 nt and differing in size by only 1 nt.

To achieve this it is necessary to have a small-pored gel and so acrylamide gels are used instead of agarose. For example, 3.5% polyacrylamide gels are used to separate DNA in the range 80-1000 nt and 12% gels to resolve fragments of between 20-100 nt. If a wide range of sizes is being analysed it is often convenient to run a gradient gel, for example, from 3.5% to 7.5%.

Sequencing gels are run the presence of denaturing agents, urea and form amide. Since it is necessary to separate DNA molecules that are very similar in size, DNA sequencing gels tend to be very long (100 cm) to maximize the separation achieved. A typical DNA sequencing gel is shown in Fig. 8.12.

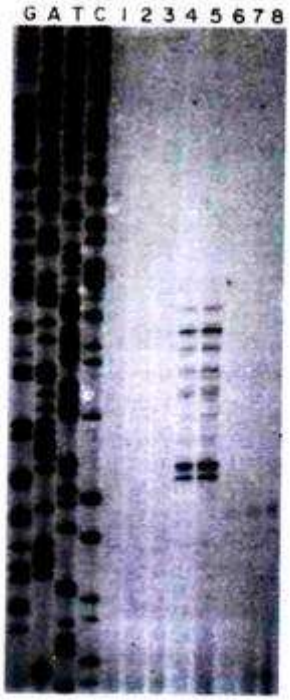


Fig. 8.12: DNA sequence gel

As mentioned above, electrophoresis in agarose can be used as a preparative method for DNA.

The DNA bands of interest can be cut out of the gel and the DNA recovered by:

(a) Electro elution,

(b) Macerating the gel piece in buffer, centrifuging and collecting the supernatant; or

(c) If low melting point agarose is used, melting the gel piece and diluting with buffer.

In each case, the DNA is finally recovered by precipitation of the supernatant with ethanol.

Polyacrylamide Gels:

Electrophoresis in acrylamide gels is frequently referred to as PAGE, being an abbreviation for Polyacrylamide gel electrophoresis. Cross-linked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of N-N-methylenebisacrylamide (normally referred to as bisacrylamide) (Fig. 8.17). Note that bis-acrylamide is basically two units of acrylamide molecules linked by a methylene group, and is used as a cross-linking agent.

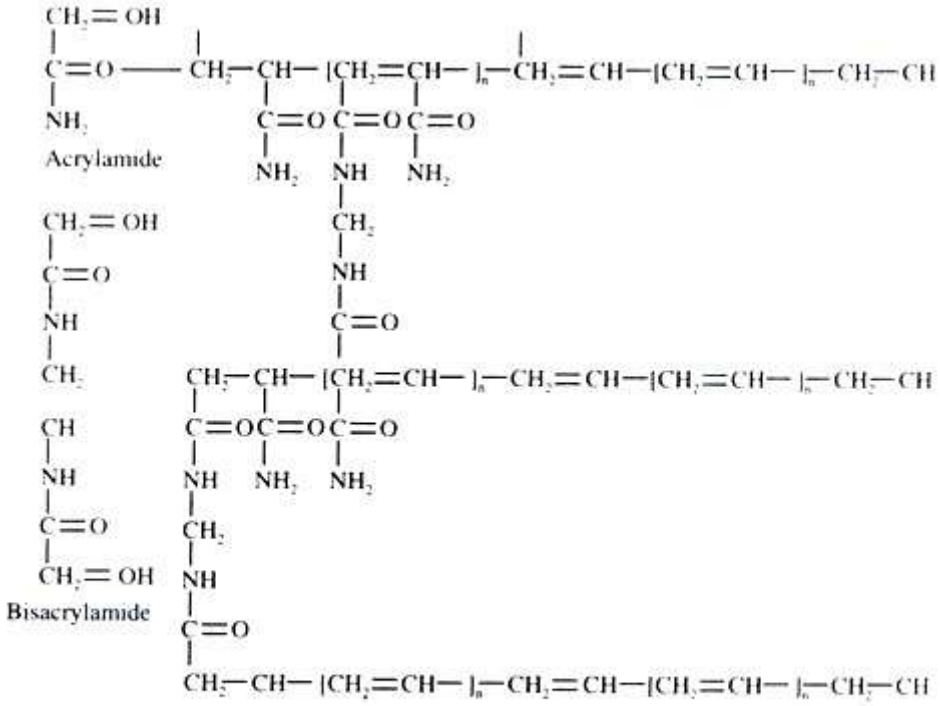


Fig. 8.17: Chemical structure of acrylamide, bisacrylamide, and polyacylamide gel

Acrylamide monomer is polymerized in a head-to-tail fashion into long chains and occasionally a bisacrylamide molecule is built into the growing chains, thus introducing a second site for chain extension. Proceeding in this way a cross-linked matrix of fairly well-defined structure is formed, the polymerization of acrylamide is an example of free radical catalysis, and is initiated by the addition of ammonium per-sulphate and the base N, N, N', N'-tetra-methylenediamine (TEMED). TEMED catalyses the decomposition of per-sulphate ion to give a free radical (i.e., a molecule with an unpaired electron):

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{\bullet}$$

If this free radical is represented as R* (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule, then the polymerization can be represented as follow:

$$R^{\bullet} + M \rightarrow RM^{\bullet}$$

$$RM^{\bullet} + M \rightarrow RMM^{\bullet}$$

$$RMM^{\bullet} + M \rightarrow RMMM^{\bullet}, \text{ etc.}$$

Free radicals are highly reactive because of the presence of an unpaired electron that needs to be paired with another electron to stabilize the molecule. R*, therefore, reacts with M, forming a single bond by sharing its unpaired electron with one from the outer shell of the monomer molecule.

In this way long chains of acrylamide are built up, being cross-linked by the introduction of the occasional bisacrylamide molecule into growing chain oxygen mops up free radicals and, therefore, all gel solutions are normally degassed (the solutions are briefly placed under vacuum to remove loosely dissolved air) prior to use.

The degassing of the gel solution also serves a second purpose. The polymerization of acrylamide is an exothermic reaction (i.e., heat is liberated) and the warming up of the gel as it sets can liberate air bubbles that become trapped in the polymerized gel. The degassing step prevents this possibility.

Photo-polymerisation is an alternative method that can be used to polymerize acrylamide gels. The ammonium per-sulphate and TEMED are replaced by riboflavin and when the gel is poured it is placed in front of a bright light for 2-3 h. Photodecomposition of riboflavin generates a free radical that initiates polymerization.

Acrylamide gels are defined in terms of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentrations of both the acrylamide and bisacrylamide. Acrylamide gels can be made with a content of between 3% and 30% acrylamide. Thus low percentage gels (e.g., 4%) have large pore sizes and are used, for example, in the electrophoresis of proteins, when free movement of the proteins by electrophoresis is required without any noticeable frictional effect and for another example, in flat-bed isoelectric focusing or the stacking gel system of an SDS-polyacrylamide gel.

Low percentage acrylamide gels are also used to separate DNA. Gels containing between 10% to 20% acrylamide are used in techniques such as SDS-gel electrophoresis, where the smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size. Proteins were originally separated on polyacrylamide gels that were polymerized in glass tubes, approximately 7 mm in diameter and about 10 cm in length. The tubes were easy to load and run, with minimum apparatus requirements. However, only one sample could be run per tube and, because conditions of separation could vary from tube to tube, comparison between different samples was not always accurate.

The later introduction of vertical gel slabs allowed running of up to 20 samples under identical conditions in a single run. Vertical slabs are now used routinely for both analysis of proteins and for the separation of DNA fragments during DNA sequence analysis. Although some workers prepare their own acrylamide gels, others purchase commercially available ready-made gels for techniques such as SDS-PAGE, native gels and isoelectric focusing (IEF).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis:

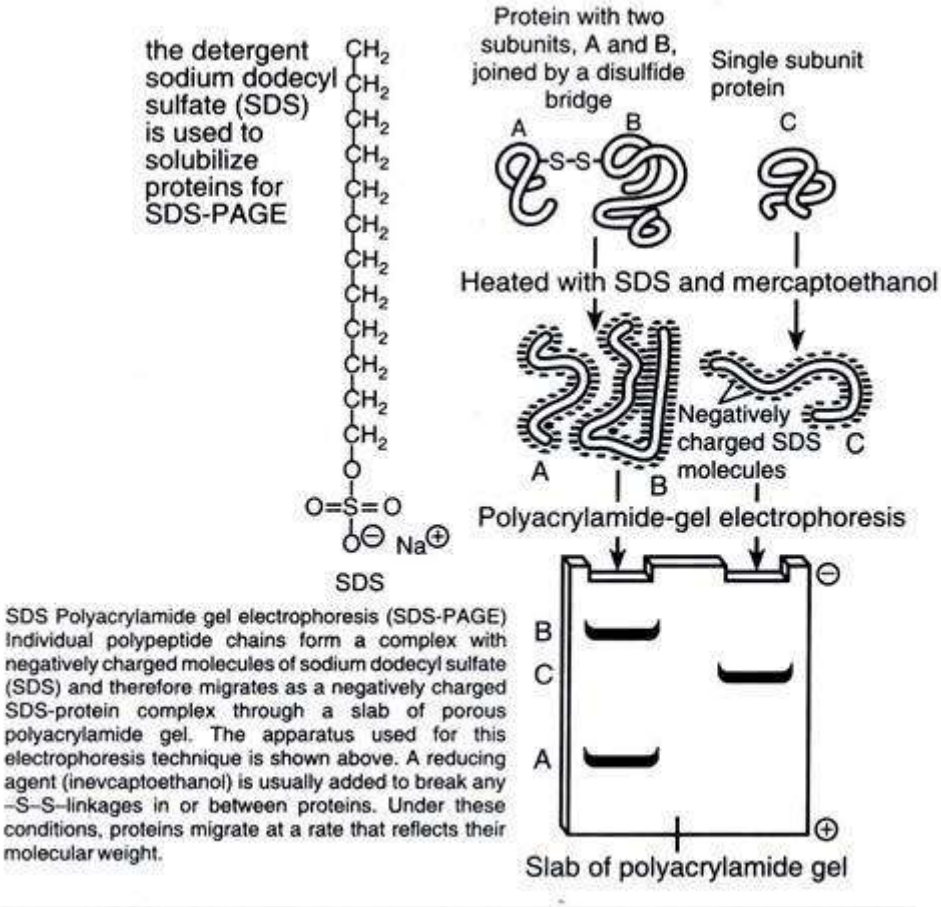
SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein

purification and, as the method is based on the separation of proteins according to size, it can also be used to determine the relative molecular mass of proteins. SDS (CH_3-$(CH_2)_{10}$-CH_2OSO_3-Na^+) is an anionic detergent.

Samples to be run on SDS-PAGE are firstly boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The beta β-mecaptoethnol reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. Each protein in the mixture is, therefore, fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain.

On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is, therefore, fully swamped by the negatively charged SDS molecules. The rod-like structure remains, as any rotation that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to rod-shape.

The sample buffer also contains an ionisable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to bottom when injected into the loading well.



SDS Polyacrylamide gel electrophoresis (SDS-PAGE) Individual polypeptide chains form a complex with negatively charged molecules of sodium dodecyl sulfate (SDS) and therefore migrates as a negatively charged SDS-protein complex through a slab of porous polyacrylamide gel. The apparatus used for this electrophoresis technique is shown above. A reducing agent (inevcaptoethanol) is usually added to break any –S–S–linkages in or between proteins. Under these conditions, proteins migrate at a rate that reflects their molecular weight.

Fig. 8.18: Principle of SDS-PAGE

Before loading to gel the protein samples are lysed to denature them by boiling them in lysis buffer for 10 min. Lysis buffer contains buffer to maintain pH along with glycerol so as to

make the protein heavy to properly settle in wells. Lysis buffer also have SDS to impart negative charge, DTT or β-mercaptoethanol to reduce the disulphide bonds in proteins.
Once the samples are all loaded, a current is passed through the gel. The samples to be separated are not in fact loaded directly into the main separating gel. When the main separating gel (normally about 5 cm long) has been poured between the glass plates and allowed to set, a shorter (approximately 0.5 cm) stacking gel is poured on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded.
The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilizing the differences in ionic strength and pH between the electrophoresis buffer and the stacking gel buffer and involves a phenomenon known as isotachophoresis.

The stacking gel has a very large pore size (4% acrylamide), which allows the proteins to move freely and concentrate, or stack, under the effect of the electric field. The band-sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have lower electrophoretic mobility than do the protein—SDS complexes, which, in turn, have lower mobility than the chloride ions (CI^-) of the loading buffer and the stacking gel buffer.
When the current is switched on, all the ionic species have to migrate at the same speed as CI^- only if they are in a region of high field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentration so that [CI^-] > [protein-SDS] > [glycinate].
There is only a small quantity of protein-SDS complexes, so they concentrate in a very tight band between glycinate and CI^- boundaries. Once the gycinate reaches the separating gel it becomes more fully ionized in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8, that of the separating gel is 8.8).
Thus, the interface between glycinate and CI^- leaves behind the protein-SDS complex's, which are left to electrophorese at their own rates. The negatively charged protein-SDS complexes now continue to move towards the anode, and as because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply the smaller the protein the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of the gels. Being a small molecule, the bromophenol blue dye is totally un-retarded and, therefore, indicates the electrophoresis front.
When the dye reaches the bottom of the gel, the current is turned off, and the gel is removed from between the glass plates and shaken in an appropriate stain solution and then washed in destain solution. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background.
A typical minigel would take about 1 h to prepare and set, 40 min to run at 200 V and have a 1 h staining time with Coomassie Brilliant Blue. Upon de-staining, strong protein bands would be seen in the gel within 10-20 min, but overnight de-staining is needed to completely remove all background stain. Vertical slab gel are invariably run, since this allows up to 10n different sample to be loaded onto a single gel. A typical SDS-polyacrylamide gel is shown in Fig. 8.19.

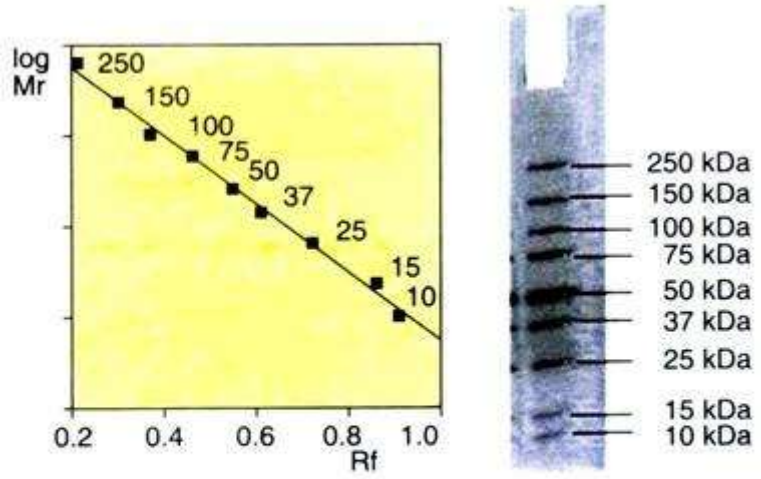


Fig. 8.19: Coomassie stained SDS-PAGE gel with separated starnds of molecular weight marker (right) and their relative mobility (left)

Typically, the separating gel used is a 15% polyacrylamide gel. This gives a gel a certain pore size in which proteins of relative molecular mass (M_r) 10,000 move through the gel relatively unhindered, whereas proteins of M_r 10, 00,000 can only just enter the pores of this gel. Gels of 15% polyacrylamide are, therefore, useful for separating proteins in the range M_r 1, 00,000 to 10,000.

However, a protein of M_r 1, 50,000, for example, would be unable to enter a 15% gel. In this case a larger-pored gel (e.g., a 10% or even 7.5% gel) would be used so that the protein could now enter the gel and be stained and identified. It is obvious; therefore, that the choice of gel to be used depends on the size of the proteins in the range of different percentage acrylamide gel is shown in Table 8.3.

Table 8.3: Percent of acrylamide required for separation of different molecular weight proteins

% Acrylamide in resolving gel		Separation size range ($M_r \times 10^{-3}$)
Single percentage:	5%	36-200
	7.5%	24-200
	10%	14-200
	12.5%	14-100*
	15%	14-60*
Gradients:	5-15%	14-200
	5-20%	10-200
	10-20%	10-150

This shows, for example, that in a 10% polyacrylamide gel proteins greater than 200 kDa in mass cannot enter the gel, whereas proteins with relative molecular mass (M_r) in the range

200,000 to 15,000 will separate. Proteins of M_r 15,000 are too small to experience the sieving effect of the gel matrix, and all run together as a single band at the electrophoresis front.
The M_r of a protein can be determined by comparing its mobility with those of a number of standard proteins of known M_r that are run on a same gel. Plotting a graph of distance moved against log M_r for each of the standard proteins, a calibration curve can be constructed. The distance moved by the protein of unknown M_r is then measured, and then its log M_r and hence M_r can be determined from the calibration curve.
SDS-gel electrophoresis is often used after each step of a purification protocol to assess the purity or otherwise of the sample. A pure protein should give a single band on an SDS-polyacrylamide gel, unless the molecule is made up of two unequal subunits. In the latter case, two bands, corresponding to the two subunits, will be seen.
Since only sub-microgram amounts of the protein are needed for the gel, very little material is used in this form of purity assessment and at the same time a value for the relative molecular mass of the protein can be determined on the same gel run (as described above), with no more material being used.

Two-Dimensional Gel Electrophoresis:

Two-dimensional electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pi); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (M_r, relative molecular weight).
Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pi, the apparent molecular weight, and the amount of each protein are obtained.
Two-dimensional electrophoresis was first introduced by P. H. O'Farrell and J. Klose in 1975. In the original technique, the first-dimension separation was performed in carrier ampholyte-containing polyacrylamide gels cast in narrow tubes. A. Gorg and colleagues developed the currently employed 2-D technique, where carrier ampholyte-generated pH gradients have been replaced with immobilized pH gradients and tube gels replaced with gels supported by a plastic backing.
A large and growing application of 2-D electrophoresis is "proteome analysis." The analysis involves the systematic separation, identification, and quantification of many proteins simultaneously from a single sample. Two-dimensional electrophoresis is used in this technique due to its unparalleled ability to separate thousands of proteins simultaneously.
Two-dimensional electrophoresis is also unique in its ability to detect post- and co-translational modifications, which cannot be predicted from the genome sequence. Applications of 2-D electrophoresis include proteome analysis, cell differentiation, and detection of disease markers, monitoring therapies, drug discovery, cancer research, purity checks, and micro-scale protein purification.
The 2-D process begins with sample preparation. Proper sample preparation is absolutely essential for a good 2-D result. The next step in the 2-D process is IPG (Isoelectric pH gradient)

strip rehydration. IPG strips are provided dry and must be rehydrated with the appropriate additives prior to IEF (Immunoelectrophoresis).
First-dimension IEF is performed on a flatbed system at very high voltages with active temperature control. Next, strip equilibration in SDS-containing buffer prepares the sample for the second-dimension separation. Following equilibration, the strip is placed on the second-dimension gel for SDS-PAGE. The final steps are visualization and analysis of the resultant two-dimensional array of spots.
2-D electrophoresis begins with 1-D electrophoresis but then separates the molecules by a second property in a direction 90 degrees from the first. In 1-D electrophoresis, proteins (or other molecules) are separated in one dimension, so that all the proteins/molecules will lie along a lane but be separated from each other by a property (e.g. isoelectric point). The result is that the molecules are spread out across a 2-D gel. Because it is unlikely that two molecules will be similar in two distinct properties, molecules are more effectively separated in 2-D electrophoresis than in 1-D electrophoresis. The two dimensions that proteins are separated into using this technique can be isoelectric point, protein complex mass in the native state, and protein mass.
To separate the proteins by isoelectric point is called isoelectric focusing (IEF). Thereby, a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pHs other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. The proteins applied in the first dimension will move along the gel and will accumulate at their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge).
For the analysis of the functioning of proteins in a cell, the knowledge of their cooperation is essential. Most often proteins act together in complexes to be fully functional. The analysis of this sub organelle organisation of the cell requires techniques conserving the native state of the protein complexes. In native polyacrylamide gel electrophoresis (native PAGE), proteins remain in their native state and are separated in the electric field following their mass and the mass of their complexes respectively. To obtain a separation by size and not by net charge, as in IEF, an additional charge is transferred to the proteins by the use of coomassie or lithium dodecyl sulfate (LDS). After completion of the first dimension the complexes are destroyed by applying the denaturing SDS-PAGE in the second dimension, where the proteins of which the complexes are composed of are separated by their mass.

Before separating the proteins by mass, they are treated with sodium dodecyl sulfate (SDS) along with other reagents (SDS-PAGE in 1-D). This denatures the proteins (that is, it unfolds them into long, straight molecules) and binds a number of SDS molecules roughly proportional to the protein's length. Because a protein's length (when unfolded) is roughly proportional to its mass, this is equivalent to saying that it attaches a number of SDS molecules roughly proportional to the protein's mass. Since the SDS molecules are negatively charged, the result of this is that all of the proteins will have approximately the same mass-to-charge ratio as each other. In addition, proteins will not migrate when they have no charge (a result of the isoelectric focusing step) therefore the coating of the protein in SDS (negatively charged) allows migration of the proteins in the second dimension (NB SDS is not compatible for use in the first dimension as it is charged and a nonionic or zwitterionic detergent needs to be used). In the

second dimension, an electric potential is again applied, but at a 90 degree angle from the first field. The proteins will be attracted to the more positive side of the gel proportionally to their mass-to-charge ratio. As previously explained, this ratio will be nearly the same for all proteins. The proteins' progress will be slowed by frictional forces. The gel therefore acts like a molecular sieve when the current is applied, separating the proteins on the basis of their molecular weight with larger proteins being retained higher in the gel and smaller proteins being able to pass through the sieve and reach lower regions of the gel.
The result of this is a gel with proteins spread out on its surface. These proteins can then be detected by a variety of means, but the most commonly used stains are silver and coomassie staining. In this case, a silver colloid is applied to the gel. The silver binds to cysteine groups within the protein. The silver is darkened by exposure to ultra-violet light. The darkness of the silver can be related to the amount of silver and therefore the amount of protein at a given location on the gel. This measurement can only give approximate amounts, but is adequate for most purposes.

Molecules other than proteins can be separated by 2D electrophoresis. In supercoiling assays, coiled DNA is separated in the first dimension and denatured by a DNA intercalator (such as ethidium bromide or the less carcinogenic chloroquine) in the second. This is comparable to the combination of native PAGE /SDS-PAGE in protein separation.

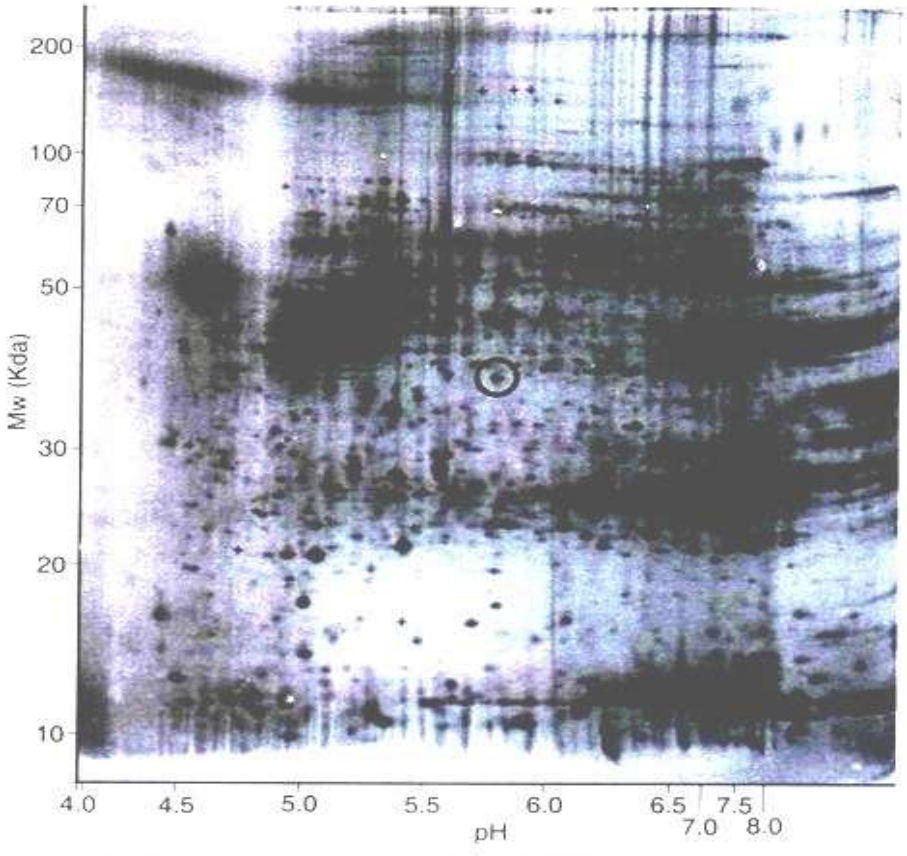


Fig. 8.28: A 2D gel (*Courtsey*: Dr D Dash, Deptt. of Biochemistry, IMS, BHU)

Silver staining is the most sensitive non-radioactive method (below 1 ng). Silver staining is a complex, multi-step process utilizing numerous reagents for which quality is critical. It is, therefore, often advantageous to purchase these reagents in the form of a dedicated kit, in which

the reagents are quality assured specifically for the silver-staining application. By omitting glutardialdehyde from the sensitizer and formaldehyde from the silver nitrate solution the method becomes compatible with mass spectrometry analysis, however, at the expense of sensitivity.

Coomassie staining, although 50- to 100-fold less sensitive than silver staining, is a relatively simple method and more quantitative than silver staining. Coomassie blue is preferable when relative amounts of protein are to be determined by densitometry. Colloidal staining methods are recommended, because they show the highest sensitivity, down to 100 ng/protein spot.

Negative Zinc—Imidazole staining has a detection limit of approx. 15 ng protein/spot and is well compatible with mass spectrometry, but it is a poor quantification technique. Fluorescent labelling and fluorescent staining with dyes have a sensitivity in-between colloidal Coomassie and Silver Staining.

These techniques require fluorescence scanners, but they are compatible with mass spectrometry and show a wide dynamic range for quantification. Apart from staining, Second-dimension gels can be blotted onto a nitrocellulose or PVDF membrane for immunochemical detection of specific proteins or chemical micro sequencing.

Preserving the Gels:

The gels are optimally stored in sheet protectors after soaking them in 10% v/v glycerol for 30 min. Un-backed gels are shrunk back to their original sizes by soaking them in 30% (v/v) methanol or ethanol/4% glycerol until they match their original sizes. For autoradiography the gels are dried onto strong filter paper with a vacuum drier or in-between two sheets of wet cellophane sealed at ends.

Further Analysis of Protein Spots:

a. Picking the spots:

Robotic systems are available that automatically picks selected protein spots from stained or de-stained gels using a pick list from the image analysis, and transfers them into micro-plates for further analysis.

b. Digestion of the proteins:

The gel plugs are automatically digested in the computer controlled Digester; the supernatant peptides are mixed with MALDI matrix material and spotted onto MALDI slides using robotic spotter.

c. MALDI-ToF mass spectrometry:

In the MALDI-ToF mass spectrometer, a laser beam is fired into the dried peptide-matrix spots for ionization of the peptides. After accurate determination of the peptide masses, databases are searched for identification of the original proteins.

Probable Questions:

1. Write basic principle of chromatography.
2. Define stationary phase and mobile phase.
3. Define absorption and partition chromatography.
4. How amino acids can be separated by paper chromatography ?
5. Write basic principle of gel filtration chromatography.
6. What is TLC? How this method can differentiate components of a mixture?
7. Write basic principle of ion exchange chromatography.
8. What are the applications of gel filtration chromatography.
9. How HPLC can resolve molecules?
10. What is forward phase and reverse phase HPLC?
11. Write basic principle of electrophoresis.
12. How agarose can resolute DNA molecule.
13. What is Polyacrylamide? How it is used in separation of protein?
14. Why SDS is used in SDS-PAGE?
15. What is the principle of SDS-PAGE?
16. What is resolving gel and stacking gel?
17. What is isoelectric focussing? How it can help to separate different proteins?
18. What is 2D PAGE? Why it is more authentic than 1D AGE in protein separation?
19. How sequencing gel are prepared?
20. How separated spots are analysed in 2D PAGE?

Suggested Readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Edition.
2. Biophysical Chemistry by Upadhyay and Upadhyay.

Unit-X
Spectroscopy: Spectrophotometer and Pesticide formulation

Objective: In this unit we will discuss spectroscopy with special emphasis to spectrophotometer. We will also discuss different modes of pesticide formulation.

Principle of Spectroscopy:

Spectroscopy is the study of the interaction of electromagnetic radiation with matter. When matter is energized (excited) by the application of thermal, electrical, nuclear or radiant energy, electromagnetic radiation is often emitted as the matter relaxes back to its original (ground) state.

The spectrum of radiation emitted by a substance that has absorbed energy is called an emission spectrum and the science is appropriately called emission spectroscopy.

Another approach often used to study the interaction of electromagnetic radiation with matter is one whereby a continuous range of radiation (e.g., white light) is allowed to fall on a substance; then the frequencies absorbed by the substance are examined.

The resulting spectrum from the substance contains the original range of radiation with dark spaces that correspond to missing, or absorbed frequencies. This type of spectrum is called an absorption spectrum. In spectroscopy the emitted or absorbed radiation is usually analyzed, i.e., separated into the various frequency components and the intensity is measured by means of an instrument called spectrometer.

The resultant spectrum is mainly a graph of intensity of emitted or absorbed radiation versus wavelength or frequency. There are in general three types of spectra: continuous, line, and band. The sun and heated solids produce continuous spectra in which the emitted radiation contains all frequencies within a region of the electromagnetic spectrum. A rainbow and light from a light bulb are examples of continuous spectra.

Line spectra are produced by excited atoms in the gas phase and contain only certain frequencies, all other frequencies being absent. Each chemical element of the periodic chart has a unique and, therefore, characteristic line spectrum. Band spectra are produced by excited molecules emitting radiation in groups of closely spaced lines that merge to form bands.

These categories of emission and absorption spectra contain tremendous amounts of useful information about the structure and composition of matter. Spectroscopy is a powerful and sensitive form of chemical analysis, as well as a method of probing electronic and nuclear structure and chemical bonding. The key to interpreting this spectral information is the knowledge that certain atomic and molecular processes involve only certain energy ranges. Fig. 12.1 shows the regions of the electromagnetic spectrum and the associated energy transitions that occur in atomic and molecular processes.

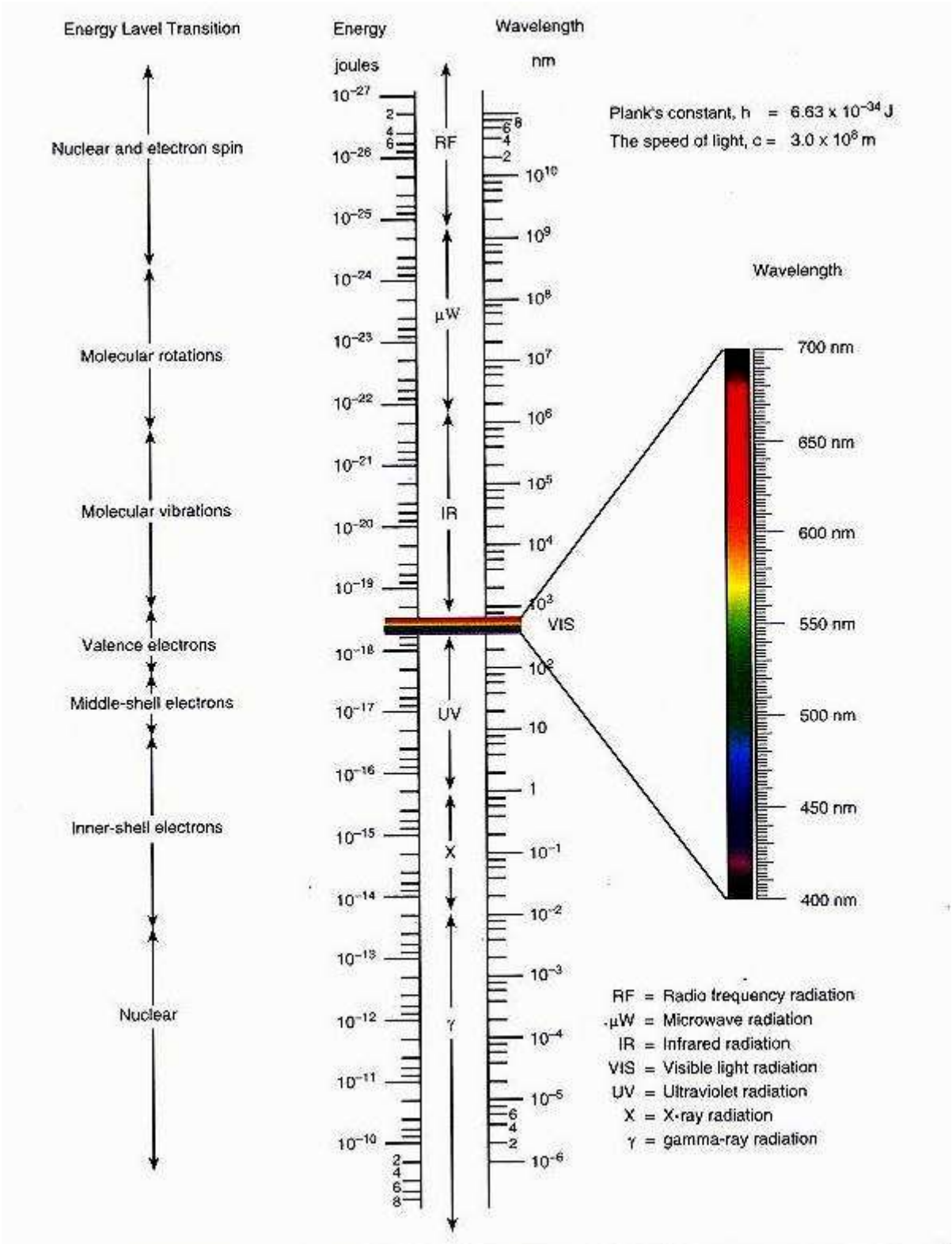


Fig. 12.1: The electromagnetic radiation ruler

Much of the scientific knowledge of the structure of the universe, from stars to atoms, is derived from interpretations of the interaction of radiation with matter. One example of the power of these techniques is the determination of the composition, the velocities, and the evolutionary dynamics of stars.

The source of the incredible amount of energy produced by the sun is nuclear fusion reactions going on within the hot interior (temperature 40 × 106 K). Two fusion cycles, the carbon cycle and the proton cycle, convert hydrogen nuclei into helium nuclei via heavier nuclei, such as carbon 12 and nitrogen 14. The enormous radiation of energy from the hot core seethes outwards by convection.

This radiation consists of the entire electromagnetic spectrum as a continuous spectrum. Towards the surface of the sun (the photosphere), the different elements all absorb at their characteristic frequencies. The radiation that shoots into space toward the earth is a continuous emission spectrum with about 22,000 dark absorption lines present in it (Fraunhofer lines), of which about 70% have been identified. These absorption lines, i.e., missing frequencies, prove that more than 60 terrestrial elements are certainly present in the sun.

Spectrophotometer:

A **spectrophotometer** can be located in many studies, biology, chemistry, and industrial laboratories. The spectrophotometer is utilized for research and data evaluation in different scientific fields.

Some of the major fields in which a spectrophotometer is employed are physics, molecular biology, chemistry, and biochemistry labs. Generally, the title refers to Ultraviolet-Visible (UV-Vis) Spectroscopy.

What a spectrophotometer does is transmit and receive light. The spectrophotometer is utilized to evaluate samples of test material by passing light by means of the sample and studying the intensity of the wavelengths.

Different samples modify the light in numerous distinct ways and this allows researchers to obtain much more facts about the check content, by viewing the change in light conduct as it passes by way of the sample.

These final results must be precise or the researcher will just be throwing away time making use of a flawed instrument. The only way to make sure accuracy is by executing a spectrophotometer calibration.

Quantification of light absorption: The chance for a photon to be absorbed by matter is given by an extinction coefficient which itself is dependent on the wavelength l of the photon. If light with the intensity I0 passes through a sample with appropriate transparency and the path length (thickness) d, the intensity I drops along the pathway in an exponential manner. The characteristic absorption parameter for the sample is the extinction coefficient a, yielding the correlation $I= I_0 e^{-\alpha d}$ .The ratio $T= I/I_0$ is called transmission. Biochemical samples usually comprise aqueous solutions, where the substance of interest is present at a molar concentration c. Algebraic transformation of the exponential correlation into an expression based on the decadic logarithm yields the law of **Beer–Lambert:**

$$\lg \frac{I_0}{I} = \lg \frac{1}{T} = \varepsilon \times c \times d = A \qquad (12.2)$$

where $[d] = 1\ \text{cm}$, $[c] = 1\ \text{mol dm}^{-3}$, and $[\varepsilon] = 1\ \text{dm}^3\ \text{mol}^{-1}\ \text{cm}^{-1}$. ε is the molar absorption coefficient (also molar extinction coefficient) ($\alpha = 2.303 \times c \times \varepsilon$). A is the absorbance of the sample, which is displayed on the spectrophotometer.

The **Beer-Lambert law (or Beer's law)** is the linear relationship between absorbance and concentration of an absorbing species. The general Beer-Lambert law is usually written as:

$$A = a(\lambda) * b * c$$

where A is the measured absorbance, $a(\lambda)$ is a wavelength-dependent absorptivity coefficient, **b** is the path length, and **c** is the analyte concentration. When working in concentration units of molarity, the Beer-Lambert lawis written as:

$$A = \varepsilon * b * c$$

where ε is the wavelength-dependent molar absorptivity coefficient with units of M^{-1} cm^{-1}. Data are frequently reported in percent transmission ($I/I_0 * 100$) or in absorbance [$A = \log (I/I_0)$]. The latter is particularly convenient. [common coefficients of near-ultraviolet absorption bands of some amino acids and nucleotides]

Sometimes the extinction coefficient is given in other units; for example,

$$A = E^{1\%} * b * c$$

where the concentration C is in gram per 100 ml of solution. This useful when the molecular weight of the solute is unknown or uncertain.

The Beer–Lambert law is valid for low concentrations only. Higher concentrations might lead to association of molecules and therefore cause deviations from the ideal behaviour. Absorbance and extinction coefficients are additive parameters, which complicates determination of concentrations in samples with more than one absorbing species. Note that in dispersive samples or suspensions scattering effects increase the absorbance, since the scattered light is not reaching the detector for readout. The absorbance recorded by the spectrophotometer is thus overestimated and needs to be corrected.

Deviations from the Beer–Lambert law

According to the Beer–Lambert law, absorbance is linearly proportional to the concentration of chromophores. This might not be the case any more in samples with high absorbance. Every spectrophotometer has a certain amount of stray light, which is light received at the detector but not anticipated in the spectral band isolated by the monochromator. In order to obtain reasonable signal-to-noise ratios, the intensity of light at the chosen wavelength (Il) should be 10 times higher than the intensity of the stray light (Istray). If the stray light gains in intensity, the effects measured at the detector have nothing or little to do with chromophore concentration. Secondly, molecular events might lead to deviations from the Beer–Lambert law. For instance, chromophores might dimerise at high concentrations and, as a result, might possess different spectroscopic parameters.

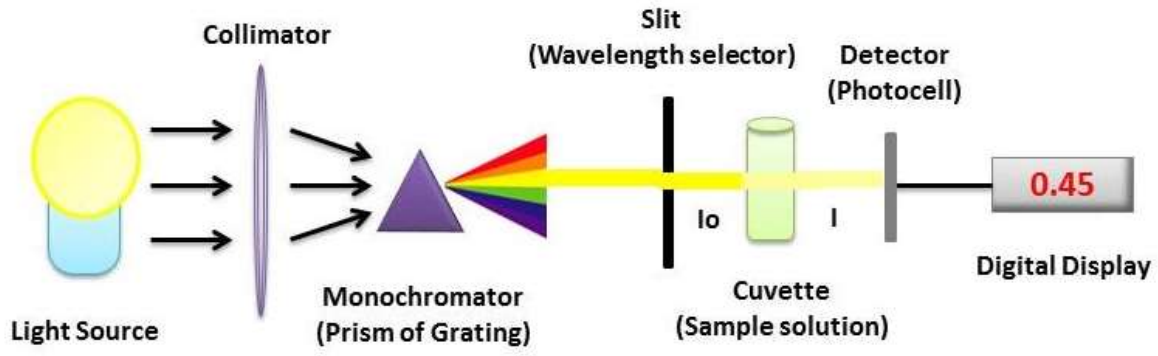
Definition: In chemistry, **spectrophotometry** is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.[2] It is more specific than the general term electromagnetic spectroscopy in that spectrophotometry deals with visible light, near-ultraviolet, and near-infrared, but does not cover time-resolved spectroscopic techniques.

Principle

The Spectrophotometer is a much more refined version of a colorimeter. In a colorimeter, filters are used which allow a broad range of wavelengths to pass through, whereas in the spectrophotometer a prism (or) grating is used to split the incident beam into different wavelengths. By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm. *The spectrophotometer* is useful for measuring the absorption spectrum of a compound, the absorption of light by a solution at each wavelength. This is the basic Principle of spectrophotometry in biochemistry.

Spectrophotometer Instrumentation

The essential components of spectrophotometer instrumentation include:



Basic Instrumentation of a Spectrophotometer

1. A Stable and cheap radiant energy source
2. A monochromator, to break the polychromatic radiation into component wavelength (or) bands of wavelengths.
3. Transport vessels (cuvettes), to hold the sample
4. A Photosensitive detector and an associated readout system

1. Radiant Energy Sources

Materials that can be excited to high energy states by a high-voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources.

a. Sources of Ultraviolet radiation: The most commonly used sources of UV radiation are the hydrogen lamp and the deuterium lamp. Xenon lamp may also be used for UV radiation, but the radiation produced is not as stable as the hydrogen lamp.

b. Sources of Visible radiation: "Tungsten filament" lamp is the most commonly used source for visible radiation. It is inexpensive and emails continuous radiation in the range between 350 and 2500nm. "Carbon arc" which provides more intense visible radiation is used in a few commercially available instruments.

c. Sources of IR radiation: "Nernst Glower" and "Global" are the most satisfactory sources of IR radiation. Global is more stable than the nearest flower.

2. Wavelength Selectors: Wavelength selectors are of two types.

1. **Filters:** "Gelatin" filters are made of a layer of gelatin, coloured with organic dyes and sealed between glass plates.
2. **Monochromators:** A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands. The essential components of a monochromator are.
    - Entrance slip-admits polychromatic light from the source
    - Collimating device–Collimates the polychromatic light onto the dispersion device.
    - Wavelength resolving device like a PRISM (or) a GRATING
    - A focusing lens (or) a mirror
    - An exit slip–allows the monochromatic beam to escape.
    -

The kinds of resolving element are of primary importance

- PRISMS
- GRATINGS

PRISMS:

A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent;

The degree of dispersion by the prism depends on upon

- The optical angle of the Prism (usually 60^0)
- The material of which it is made

Two types of Prisms are usually employed in commercial instruments. Namely, 60^0 cornu quartz prism and 30^0 Littrow Prism.

GRATINGS:

Gratings are often used in the monochromators of spectrophotometers operating ultraviolet, visible and infrared regions.

3. Sample Containers

Sample containers are also one of the parts of Spectrophotometer instrumentation. Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as "CUVETTES".

Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes Quartz. Most of the spectrophotometric studies are made in solutions, the solvents assume prime importance.

The most important factor in choosing the solvent is that the solvent should not absorb (optically transparent) in the same region as the solute.

4. Detection Devices

Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it. Important requirements for a detector including

- High sensitivity to allow the detection of low levels of radiant energy
- Short response time

- Long-term stability
- An electric signal which easily amplified for a typical readout apparatus.

5. Amplification And Readout

Radiation detectors generate electronic signals which are proportional to the transmitted light. These signals need to be translated into a form easy to interpret. This is accomplished by using amplifiers, Ammeters, Potentiometers and Potentiometric recorders.

The above 5 major parts are the major part of Spectrophotometer instrumentation. Now let us see the Applications of Spectrophotometer.

Spectrophotometer Applications

How to use the spectrophotometer? There are uses of spectrophotometry in biochemistry which are listed below:

1. Qualitative Analysis

The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations. This is done by plotting absorption spectrum curves. Absorption by a compound in different regions gives some hints to its structure.

2. Quantitative Analysis

Spectrophotometer uses in the Quantitative analysis of Biochemistry practicals. Quantitative analysis method developing for determining an unknown concentration of a species by absorption spectrometry.

Most of the organic compounds of biological interest absorb in the UV-visible range of the spectrum.

Thus, several important classes of biological compounds may be measured semi-quantitatively using the UV-visible spectrophotometer. Nucleic acids at 254nm protein at 280nm provide good examples of such use.

The absorbance at 280nm by proteins depends on their "Tyrosine" and "Tryptophan" content.

- **Estimation of Proteins by Lowry method**
- **Estimation of Tyrosine by Folin-Ciocalteau Method**
- **Estimation of Blood Glucose level by Folin-Wu method**

3. Enzyme Assay:

This is the basic application of spectrophotometry. This assay is carried out most quickly and conveniently when the substrate (or) the product is color (or) absorbs light in the UV range.

Eg 1: Lactate Dehydrogenase (LDH)

Lactate + NAD $^+$ ↔ Pyruvate + NADH + H$^+$

- The LDH is engaged in the transfer of electrons from lactate to NAD$^+$.
- The products of the reaction are pyruvate, NAD, and a proton
- One of the products, NADH, **absorbs radiation in the UV range at 340 nm** while its oxidized counterpart, NAD$^+$ does not.

- The reaction in the forward direction can be followed by measuring the increment in the light absorption of the system at 540nm in a spectrophotometer.

Eg 2: Pyruvate Kinase
Phosphoenolpyruvate + ADP ↔ Pyruvate + ATP
Pyruvate + NADH + H^+ ↔ Lactate + NAD^+
We have added a large excess of NADH to the system, the system now absorbs at 340nm. According to the above-given reactions, each molecule of Pyruvate formed in the reaction, **a molecule of NADH is oxidized to NAD+** in the second reaction when the system converts pyruvate to locate. Since NAD+ does not absorb at 340nm, the absorbance goes on decreasing with increased pyruvate generation. Such measurements are known as "Coupled assays".
Sample enzymatic assays:

- **Assay of Urease Enzyme Activity**
- **Assay of Salivary Amylase enzyme activity**
- **Effect of Temperature on Amylase activity**

4. Molecular Weight Determination
Molecular weights of amine picrates, sugars and much aldehyde and ketone compounds have been determined by this method. Molecular weights of only small molecules may be determined by this method.

1. **Study of Cis-Trans Isomerism:** Geometrical isomers differ in the spatial arrangement of groups about a plane, the absorption spectra of the isomers also differs. The trans-isomer is usually more elongated than its cis counterpart. Absorption spectrometry can be utilized to study Cis-Trans isomerism.
2. **Control of Purification:** Impurities in a compound can be detected very easily by spectrophotometric studies. "Carbon disulfide" impurity in carbon tetrachloride can be detected easily by measuring absorbance at 318nm where carbon sulfide absorbs. A lot many commercial solutions are routinely tested for purity spectroscopically.

5. Other Physiochemical Studies:
Spectrophotometry (UV-VIS) has been used to study the following physiochemical phenomena:

- Heats of formation of molecular addition compound and complexes in solution
- Determination of the empirical formula
- Formation constants of complexes in solution
- Hydration equilibrium of carbonyl compounds
- Association constants of weak acids and bases in organic solvents
- Protein-dye interactions
- Chlorophyll-Protein complexes
- Vitamin-A aldehyde–Protein complex
- Determination of reaction rates
- Dissociation constants of acids and bases
- Association of cyanine dyes

These are the basic spectrophotometer instrumentation and its applications.

PESTICIDE FORMULATION

Pesticide chemicals in their "raw" or unformulated state are not usually suitable for pest control. These concentrated chemicals and active ingredients may not mix well with water, may be chemically unstable, and may be difficult to handle and transport. For these reasons, inert substances, such as clays and solvents, are added to improve application effectiveness, safety, handling, and storage. Inert ingredients do not possess pesticidal activity and are added to serve as a carrier for the active ingredient. The percentage of inert ingredients are listed in the formulation or designate them as "other ingredients" on the labels of the formulation. There are several inert substances, such as petroleum distillates and xylene, which have a specific statement identifying their presence in the formulation.

The mixture of active and inert ingredients is called a pesticide formulation. This formulation may consist of:

• The pesticide active ingredient that controls the target pest.

• The carrier, such as an organic solvent or mineral clay.

• Adjuvants, such as stickers and spreaders.

• Other ingredients, such as stabilizers, dyes and chemicals that improve or enhance pesticidal activity.

Usually a formulated product is mixed with water or oil for final application. Most baits, granules, gels, and dusts, however, are ready for use without additional dilution.

A single active ingredient often is sold in several kinds of formulations. Abbreviations are frequently used to describe the formulation (e.g., WP for wettable powders); how the pesticide is used (e.g., TC for termiticide concentrate); or the characteristics of the formulation (e.g., ULV for an ultra-low-volume formulation). For example, an 80% SP contains 80 percent by weight of active ingredient and is a "soluble powder." If it is in a 10-pound bag, it contains 8 pounds of a.i. and 2 pounds of inert ingredient. Liquid formulations indicate the amount of a.i. in pounds per gallon. For example, 1E means 1 pound, and 4E means 4 pounds of the a.i. per gallon in an emulsifiable concentrate formulation. If more than one formulation is available for pest control, the best one is chosen for the job.

Types of formulations:

1) Liquid Formulations

Liquid formulations are generally mixed with water, but in some instances crop oil, diesel fuel, kerosene, or some other light oil are also mixed as a carrier. Common liquid pesticide formulations are –

a) Emulsifiable Concentrates (EC or E)

An emulsifiable concentrate formulation usually contains a liquid active ingredient, one or more petroleum-based solvents (which give EC formulations their strong odor), and an agent—known as an emulsifier—that allows the formulation to be mixed with water to form an emulsion. Upon mixing with water, they take on a "milky" appearance Most ECs contain

between 25% and 75% (2–8 pounds) active ingredient per gallon. ECs are among the most versatile formulations. They are used against agricultural, ornamental and turf, forestry, structural, food processing, livestock, and public health pests. They are adaptable to many types of application equipment including portable sprayers, hydraulic sprayers, low-volume ground sprayers, mist blowers, and low-volume aircraft sprayers.

Advantages of emulsifiable concentrates:

- Relatively easy to handle, transport, and store
- Little agitation required; will not settle out or separate when equipment is running
- Not abrasive
- Will not plug screens or nozzles
- Little visible residue on treated surfaces

Disadvantages of emulsifiable concentrates:

- High a.i. concentration makes it easy to overdose or underdose through mixing or calibration errors.
- Easily absorbed through skin of humans or animals.
- Solvents may cause rubber or plastic hoses, gaskets, and pump parts and surfaces to deteriorate.
- May cause pitting or discoloration of painted finishes.
- Flammable—should be used and stored away from heat or open flame.
- May be corrosive.

b) Solutions (S) :

Some pesticide active ingredients dissolve readily in a liquid carrier, such as water or a petroleum-based solvent. When mixed with the carrier, they form a solution that does not settle out or separate. Formulations of these pesticides usually contain the active ingredient, the carrier, and one or more other ingredients.

c) Ready-to-Use Low Concentration Solutions (RTU) :

Low-concentrate RTU formulations are ready to use and require no further dilution before application (Figure 6). They consist of a small amount of active ingredient (often 1% or less per unit volume) dissolved in an organic solvent. They usually do not stain fabrics nor have unpleasant odours. They are especially useful for structural and institutional pests and for household use. Major disadvantages of low-concentrate formulations include limited availability and high cost per unit of active ingredient.

d) Ultra-Low Volume (ULV) :

These concentrates may approach 100% active ingredient. They are designed to be used “as is” or to be diluted with only small quantities of a specified carrier. They are used at rates of no more than 1/2 gallon per acre. These special purpose formulations are used mostly in outdoor applications, such as in agricultural, forestry, ornamental, and mosquito control programs.

Advantages of ultra-low-volume formulations:

- Relatively easy to transport and store Remain in solution; little agitation required.
- Not abrasive to equipment.
- Will not plug screens and nozzles.
- Leave little visible residue on treated surfaces.

Disadvantages:

- Difficult to keep pesticide on target—high drift hazard.
- Specialized equipment required.
- Easily absorbed through skin of humans or animals.
- Solvents may cause rubber or plastic hoses, gaskets, and pump parts and surfaces to deteriorate.

• Calibration and application must be done very carefully because of the high concentration of active ingredient

e) Invert Emulsions :

An invert emulsion contains a water-soluble pesticide dispersed in an oil carrier. Invert emulsions require a special kind of emulsifier that allows the pesticide to be mixed with a large volume of petroleum-based carrier, usually fuel oil. Invert emulsions aid in reducing drift. With other formulations, some spray drift results when water droplets begin to evaporate before reaching target surfaces; as a result, the droplets become very small and light. Because oil evaporates more slowly than water, invert emulsion droplets shrink less; therefore, more pesticide reaches the target. The oil helps to reduce runoff and improves rain resistance. It also serves as a sticker-spreader by improving surface coverage and absorption. Because droplets are relatively large and heavy, it is difficult to get thorough coverage on the undersides of foliage. Invert emulsions are most commonly used along rights-of-way where drift to susceptible nontarget plants or sensitive areas can be a problem.

2. Flowables (F)/Liquids (L)

A flowable or liquid formulation combines many of the characteristics of emulsifiable concentrates and wettable powders. Manufacturers use these formulations when the active ingredient is a solid that does not dissolve in either water or oil. The active ingredient, impregnated on a substance such as clay, is ground to a very fine powder. The powder is then suspended in a small amount of liquid. The resulting liquid product is quite thick (Figure 8). Flowables and liquids share many of the features of emulsifiable concentrates, and they have similar disadvantages. They require moderate agitation to keep them in suspension and leave visible residues similar to those of wettable powders. Flowables/liquids are easy to handle and apply. Because they are liquids, they are subject to spilling and splashing. They contain solid particles, so they contribute to abrasive wear of nozzles and pumps. Flowable and liquid suspensions settle out in their containers. Always shake them thoroughly before pouring and mixing. Because flowable and liquid formulations tend to settle, manufacturers package them in containers of 5 gallons or less to make remixing easier.

3. Aerosols (A)

These formulations contain one or more active ingredients and a solvent. Most aerosols contain a low percentage of active ingredients. There are two types of aerosol formulations: the ready-to-use type commonly available in pressurized, sealed containers and those products used in electric- or gasoline-powered aerosol generators that release the formulation as a "smoke" or "fog." Ready-to-use aerosols are usually small, self-contained units that release the pesticide when the nozzle valve is triggered. The pesticide is driven through a fine opening by an inert gas under pressure, creating fine droplets. These products are used in greenhouses, in small areas inside buildings, or in localized outdoor areas. Commercial models, which hold 5–10 pounds of pesticide, are usually refillable.

Advantages :

- Ready to use
- Portable
- Easily stored
- Convenient way to buy a small amount of a pesticide
- Retain potency over fairly long time

Disadvantages:

- Practical for only very limited uses
- Risk of inhalation injury
- Hazardous if punctured, overheated, or used near an open flame
- Difficult to confine to target site or pest

Formulations for smoke or fog generators are aerosol formulations but not under pressure. They are used in machines that break the liquid formulation into a fine mist or fog (aerosol) using a rapidly whirling disk or heated surface. These formulations are used mainly for insect control in structures such as greenhouses and warehouses and for mosquito and biting fly control outdoors.

4. Liquid Baits

An increasing number of insecticides and rodenticides are being formulated as liquid baits. Liquid rodenticides are mixed with water and placed in bait stations designed for these products. They have two major benefits. Liquid rodenticides are effective in controlling rodents, especially rats, in areas where they cannot find water. They are also effective in areas of poor sanitation where readily available food renders traditional baits ineffective.

Liquid insecticide baits are used primarily by the structural pest control industry for controlling ants and, to a lesser extent, cockroaches. They are packaged as ready-to-use, sugar-based liquids placed inside bait stations. Liquid insecticide ant baits have a number of advantages. They are very effective against certain species of sugar-feeding ants. These ants typically accept and transfer liquid baits into the ant colonies. However, some ants will not feed on liquid baits. Liquid baits also must be replaced often.

5. Dry or Solid Formulations

Dry formulations can be divided into two types: ready-to-use and concentrates that must be mixed with water to be applied as a spray. This section will present more detailed information about the common dry or solid pesticide formulations.

6. Dusts (D)

Most dust formulations are ready to use and contain a low percentage of active ingredients (usually 10% or less by weight), plus a very fine, dry inert carrier made from talc, chalk, clay, nut hulls, or volcanic ash. The size of individual dust particles varies. A few dust formulations are concentrates and contain a high percentage of active ingredients. These concentrates are mixed with dry inert carriers before applying. Dusts are always used dry and can easily drift to non target sites. They are widely used as seed treatments and sometimes for agricultural applications. In structures, dust formulations are used in cracks and crevices and for spot treatments to control insects such as cockroaches. Insects ingest poisonous dusts during grooming or absorb the dusts through their outer body covering. Dusts also are used to control lice, fleas, and other parasites on pets and livestock.

Advantages of dust formulations :

- Most are ready to use, with no mixing .
- Effective where moisture from a spray might cause damage.
- Require simple equipment.
- Effective in hard-to-reach indoor areas.

Disadvantages:

- Easily drift off target during application.
- Residue easily moved off target by air movement or water.
- May irritate eyes, nose, throat, and skin.
- Will not stick to surfaces as well as liquids.
- Dampness can cause clogging and lumping.
- Difficult to get an even distribution of particles on surfaces.

Special dusts, known as tracking powders, are used for monitoring and controlling rodents and insects. For rodent control, the tracking powder consists of finely ground dust combined with a stomach poison. Rodents walk through the dust, pick it up on their feet and fur, and ingest it when they clean themselves. Tracking powders are useful when bait acceptance is poor because of an abundant, readily available food supply. Nontoxic powders, such as talc or flour, often are used to monitor and track the activity of rodents in buildings.

7. Baits (B)

A bait formulation is an active ingredient mixed with food or another attractive substance. The bait either attracts the pests or is placed where the pests will find it. Federal regulations require that certain rodenticide baits must be contained in tamper-resistant bait stations. Pests are killed by eating the bait that contains the pesticide. The amount of active ingredient in most bait formulations is quite low, usually less than 5%.

Baits are used inside buildings to control ants, roaches, flies, other insects, and rodents. Outdoors they sometimes are used to control snails, slugs, and insects such as ants and termites. Their main use is for control of vertebrate pests such as rodents, other mammals, and birds.

Advantages of baits :

- Entire area need not be covered because pest goes to bait
- Control pests that move in and out of an area

Disadvantages:

- Can be attractive to children and pets
- May kill domestic animals and nontarget wildlife outdoors
- Pest may prefer the crop or other food to the bait
- Dead vertebrate pests may cause odor problem
- Other animals may be poisoned as a result of feeding on the poisoned pests
- If baits are not removed when the pesticide becomes ineffective, they may serve as a food supply for the target pest or other pests
- Laws require that outdoor, above-ground placement of certain rodenticide bait products be contained in tamper-resistant bait stations

Pastes and gels are mainly used in the pest control industry for ants and cockroaches. Insecticides formulated as pastes and gels are now the primary formulations used in cockroach control. They are designed to be injected or placed as either a bead or dot inside small cracks and crevices of building elements where insects tend to hide or travel. Two basic types of tools are used to apply pastes and gels: syringes and bait guns. The applicator forces the bait out of the tip of the device by applying pressure to a plunger or trigger.

8. Granules (G)

Granular formulations are similar to dust formulations except granular particles are larger and heavier. The coarse particles are made from materials such as clay, corncobs, or walnut shells. The active ingredient either coats the outside of the granules or is absorbed into them. The amount of active ingredient is relatively low, usually ranging from less than 1 to 15 percent by weight.

Granular pesticides are most often used to apply chemicals to the soil to control weeds, fire ants, nematodes, and insects living in the soil or for absorption into plants through the roots. Granular formulations are sometimes applied by airplane or helicopter to minimize drift or to penetrate dense vegetation. Once applied, granules release the active ingredient slowly. Some granules require soil moisture to release the active ingredient. Granular formulations also are used to control larval mosquitoes and other aquatic pests. Granules are used in agricultural, structural, ornamental, turf, aquatic, right-of-way, and public health (biting insect) pest control operations.

Advantages of granular formulations:

- Ready to use, no mixing
- Drift hazard is low, and particles settle quickly

• Little hazard to applicator; no spray, little dust
• Weight carries the formulation through foliage to soil or water target
• Simple application equipment needed, such as seeders or fertilizer spreaders
• May break down more slowly than WPs or ECs because of a slow-release coating

Disadvantages:
• Often difficult to calibrate equipment and apply uniformly
• Will not stick to foliage or other uneven surfaces
• May need to be incorporated into soil or planting medium
• May need moisture to activate pesticide
• May be hazardous to non target species, especially waterfowl and other birds that mistakenly feed on the seed-like granules
• May not be effective under drought conditions because the active ingredient is not released in sufficient quantity to control the pest.

9. Pellets (P or PS)

Most pellet formulations are very similar to granular formulations; the terms often are used interchangeably. In a pellet formulation, however, all the particles are the same weight and shape. The uniformity of the particles allows use with precision application equipment. A few fumigants are formulated as pellets; some may be referred to as tablets. However, these are clearly labelled as fumigants. Do not confuse them with nonfumigant pellets.

10. Wettable Powders (WP or W)

Wettable powders are dry, finely ground formulations that look like dusts. They usually must be mixed with water for application as a spray. A few products, however, may be applied either as a dust or as a wettable powder; the choice is left to the applicator. Wettable powders contain 5%–95% active ingredient by weight, usually 50% or more. The particles do not dissolve in water. They settle out quickly unless constantly agitated to keep them suspended. Wettable powders are one of the most widely used pesticide formulations. They can be used for most pest problems and in most types of spray equipment where agitation is possible. Wettable powders have excellent residual activity. Because of their physical properties, most of the pesticide remains on the surface of treated porous materials such as concrete, plaster, and untreated wood. In such cases, only the water penetrates the material.

Advantages of wettable powders:

• Easy to store, transport, and handle
• Less likely than ECs and other petroleum-based pesticides to cause unwanted harm to treated plants, animals, and surfaces
• Easily measured and mixed
• Less skin and eye absorption than ECs and other liquid formulations

Disadvantages:
• Inhalation hazard to applicator while measuring and mixing the concentrated powder
• Require good and constant agitation (usually mechanical) in the spray tank or will quickly settle out if the agitator is turned off

• Abrasive to many pumps and nozzles, causing them to wear out quickly

11. Soluble Powders (SP or WSP)

Soluble powder formulations look like wettable powders. However, when mixed with water, soluble powders dissolve readily and form a true solution. After they are mixed thoroughly, no additional agitation is necessary. The amount of active ingredient in soluble powders ranges from 15% to 95% by weight; it usually is more than 50%. Soluble powders have all the advantages of wettable powders and none of the disadvantages except the inhalation hazard during mixing. Few pesticides are available in this formulation because few active ingredients are readily soluble in water.

12. Water-Dispersible Granules (WDG) or Dry Flowables (DF)

Water-dispersible granules, also known as dry flowables, are like wettable powders except instead of being dustlike, they are formulated as small, easily measured granules. Water-dispersible granules must be mixed with water to be applied. Once in water, the granules break apart into fine particles similar to wettable powders. The formulation requires constant agitation to keep them suspended in water. The percentage of active ingredient is high, often as much as 90 percent by weight. Water-dispersible granules share many of the same advantages and disadvantages of wettable powders except:

• They are more easily measured and mixed

• Because of low dust, they cause less inhalation hazard to the applicator during handling

13. Other Formulations

Other formulations include chemicals that cannot be clearly classified as liquid or as dry/solid pesticide formulations. These are -

a. Microencapsulated Materials (M or ME)

Manufacturers cover liquid or dry pesticide particles in a plastic coating to produce a microencapsulated formulation. Microencapsulated pesticides are mixed with water and sprayed in the same manner as other sprayable formulations. After spraying, the plastic coating breaks down and slowly releases the active ingredient. Microencapsulated materials have several advantages:

• Highly toxic materials are safer for applicators to mix and apply

• Delayed or slow release of the active ingredient prolongs its effectiveness, allowing for fewer and less precisely timed applications

• The pesticide volatilizes more slowly; less is lost from the application site

Microencapsulated materials offer fewer hazards to the skin than ordinary formulations. Microencapsulated materials, however, pose a special hazard to bees. Foraging bees may carry microencapsulated materials back to their hives because they are about the same size as pollen grains. As the capsules break down, they release the pesticide, poisoning the adults and brood. Breakdown of the microencapsulated materials to release the pesticide sometimes depends on weather conditions. Under certain conditions, the microencapsulated materials may break down more slowly than expected. This could leave higher residues of pesticide active ingredient in treated areas beyond normal restricted-entry or harvest intervals with the potential

to injure fieldworkers. For this reason, regulations require long restricted-entry intervals for some microencapsulated formulations.

b. Water-Soluble Packets (WSB or WSP)

Water-soluble packets reduce the mixing and handling hazards of some highly toxic pesticides. Manufacturers package precise amounts of wettable powder or soluble powder formulations in a special type of plastic bag. When you drop these bags into a filled spray tank, they dissolve and release their contents to mix with the water. There are no risks of inhaling or contacting the undiluted pesticide as long as you do not open the packets. Once mixed with water, however, pesticides packaged in water-soluble packets are no safer than other diluted pesticides.

c. Attractants

Attractants include pheromones, sugar and protein syrups, yeasts, and rotting meat. Pest managers use these attractants in various types of traps (Figure 19). Attractants also can be combined with pesticides and sprayed onto foliage or other items in the treatment area.

d. Impregnated Products

Manufacturers impregnate (saturate) pet collars, livestock ear tags, adhesive tapes, plastic pest strips, and other products with pesticides (Figure 20). These pesticides evaporate over time, and the vapors provide control of nearby pests. Some paints and wood finishes have pesticides incorporated into them to kill insects or retard fungal growth. Fertilizers also may be impregnated with pesticides.

e. Repellents

Various types of insect repellents are available in aerosol and lotion formulations. People apply these to their skin or clothing or to plant foliage to repel biting and nuisance insects. You can mix o ther types of repellents with water and spray them onto ornamental plants and agricultural crops to prevent damage from deer, dogs, and other animals.

f. Animal Systemics

Systemic pesticides protect animals against fleas and other external blood-feeding insects as well as against worms and other internal parasites. A systemic animal pesticide is one that is absorbed and moves within the animal. These pesticides enter the animal’s tissues after being applied orally or externally. Oral applications include food additives and premeasured capsules and liquids. External applications involve pour-on liquids, liquid sprays, and dusts.

g. Fumigants

Fumigants are pesticides that form gases or vapours toxic to plants, animals, and microorganisms. Some active ingredients are formulated, packaged, and released as gases; others are liquids when packaged under high pressure and change to gases when they are released. Other active ingredients are volatile liquids when enclosed in an ordinary container and, therefore, are not formulated under pressure. Others are solids that release gases when applied under conditions of high humidity or in the presence of water vapor. Fumigants are used for structural pest control, in food and grain storage facilities, and in regulatory pest

control at ports of entry and at state and national borders. In agricultural pest control, fumigants are used in soil, greenhouses, granaries, and grain bins.

Advantages of fumigants:
• Toxic to a wide range of pests
• Can penetrate cracks, crevices, wood, and tightly packed areas such as soil or stored grains
• Single treatment usually kills most pests in treated area

Disadvantages of fumigants:
• The target site must be enclosed or covered to prevent the gas from escaping
• Nonspecific in that they are highly toxic to humans and all other living organisms
• Require the use of specialized protective equipment, including respirators specifically approved for use with fumigants
• Require the use of specialized application equipment

h. Adjuvants

Adjuvants are substances used with a pesticide to enhance performance. By themselves, they do not possess pesticidal activity. Adjuvants may be added to the product at the time of formulation or by the applicator to the spray mix just prior to treatment. Adjuvants include surfactants, compatibility agents, antifoaming agents and spray colorants (dyes), and drift control agents.

Care should be taken when selecting an adjuvant. Pesticide performance can differ depending on what type of adjuvant is used. The pesticide label will state if specific surfactants are required and the amount (%) of active ingredient it must contain.

Glossary related to pesticide formulation:

Abrasive: Capable of wearing away or grinding down another object.
Active ingredient (a.i.): The substance in a pesticide product that is intended to kill, repel, or otherwise control a target pest.
Adjuvant: Chemical that is either premixed in the pesticide formulation or added to the spray tank to improve mixing, application or to enhance pesticidal activity.
Carrier: The primary material used to allow a pesticide to be dispersed effectively. For example, talc in a dust formulation.
Diluent: Anything used to dilute a pesticide.
Emulsifier: Agent that helps to prevent an emulsion from separating.
Emulsion: A mixture of two or more liquids that is not soluble in one another. For example, oil droplets dispersed in water.
Formulation: A mixture of active ingredient combined during manufacture with inert ingredients.
Inert ingredients: All materials in the pesticide formulation other than the active ingredient. They are added to dilute the pesticide or to make it safer, more effective, and easier to measure, mix, apply, and handle. Some inert ingredients may be toxic or hazardous to people.
Insoluble: Does not dissolve in liquid.
Phytotoxicity: Injury to plants.
Soluble: Able to dissolve in another substance, usually a liquid.

Solvent: A liquid, such as water, kerosene, xylene, or alcohol, that will dissolve a pesticide to form a solution.
Suspension: A substance that contains undissolved particles mixed throughout a liquid.
Volatile: Evaporating rapidly; turning easily into a gas or vapor.

Probable Questions:

1. What is the basic principle of spectroscopy?
2. Explain Lambert-Beer law.
3. What is molar extinction coefficient? How it is calculated?
4. Describe different components of a spectrophotometer?
5. Describe different applications of spectrophotometer?
6. What are the advantages and disadvantages of emulsifiable pesticides?
7. What is aerosol pesticide? What are its advantages and disadvantages ?
8. What are the advantages and disadvantages of dust pesticide formulation ?
9. What are the advantages and disadvantages of bait pesticide formulation ?
10. What are the advantages and disadvantages of granular pesticide formulation ?
11. What are the advantages and disadvantages of wettable pesticide formulation ?
12. What are the advantages and disadvantages of fumigant pesticide formulation ?
13. What is adjuvant? How it is used as pesticide formulation ?

Suggested Readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7^{th} Edition.
2. Biophysical Chemistry by Upadhyay and Upadhyay.

Unit-XI

Blotting Methods: Southern, Northern & Western Blotting

Objective: In this unit we will discuss different types of blotting techniques such as Southern Blotting for DNA, Northern Blotting for RNA and Western Blotting for Proteins.

I. Southern Blotting:

Developed by E.M. Southern, the technique of Southern blotting is one of the most important methods used in molecular biology. In Southern blotting, DNA is transferred from a gel to a membrane for hybridization analysis.

Principle:

- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/ Steps

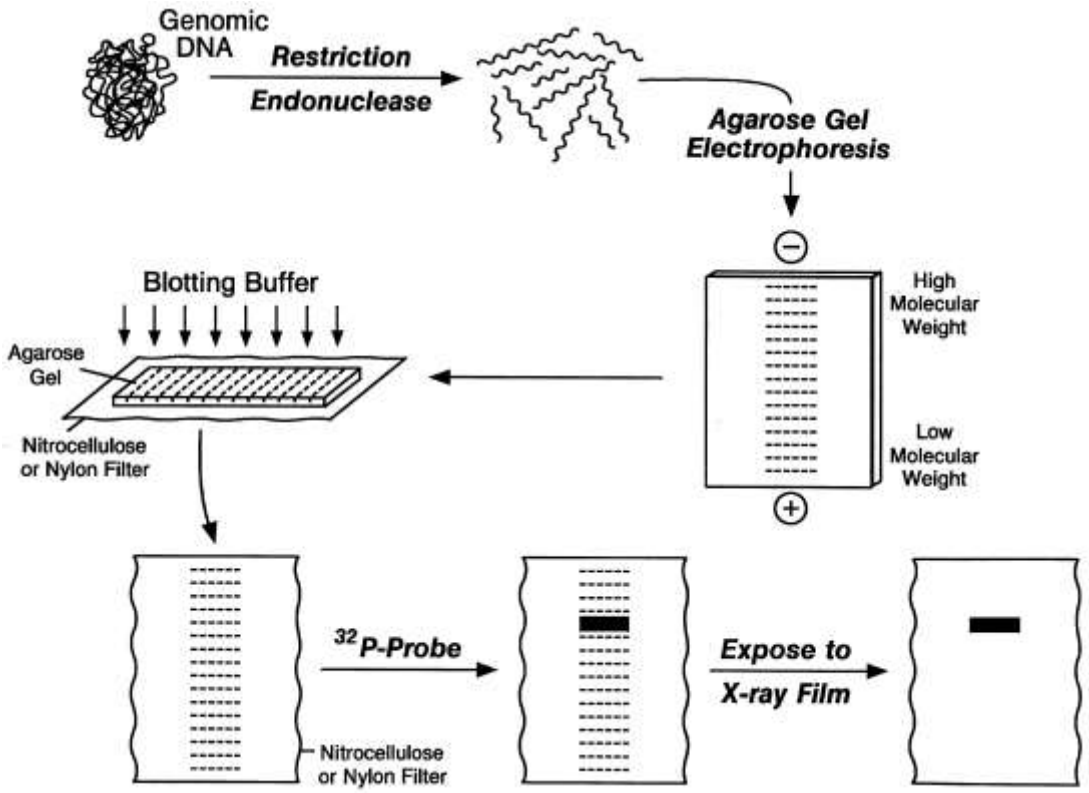
1. Restriction digest: by RE enzyme and amplification by PCR
2. Gel electrophoresis: SDS gel electrophoresis
3. Denaturation: Treating with HCl and NaOH
4. Blotting
5. Baking and Blocking with casein in BSA
6. Hybridization using labelled probes
7. Visualization by autoradiogram

Step I: Restriction digest

- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments
- The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis



Step III: Denaturation

- The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.
- DNA strands get separated

Step IV: Blotting

- The separated strands of DNA is then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

- The DNA bound to membrane is then treated with labelled probe
- The labelled probe contains the complementary sequences to the gene of interest
- The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Application of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting
3. Used for paternity testing, criminal identification, victim identification
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism
6. To identify mutation or gene rearrangement in the sequence of DNA
7. Used in diagnosis of disease caused by genetic defects
8. Used to identify infectious agents

Significance:

Southern blotting is useful for detecting major gene arrangements. This technique plays important role in DNA finger print, identification of novel gene, identification of structurally related genes in the species etc.

II. Northern Blotting:

Northern blotting is a technique used to analyse RNA. In northern blot, RNA is transferred from agarose gel to nitrocellulose paper for hybridization analysis. Total cellular RNA or poly (A) RNA, is separated by size on an agarose gel. The RNA molecules in the gel can be transferred to nitrocellulose or nylon membrane.

The RNA molecules are separated on agarose gel containing formaldehyde or dimethylsulfoxide. The formaldehyde is used to alter secondary structure of RNA molecules. Nitrocellulose filter paper binds strongly to denatured RNA, but not with RNA having secondary structure. The nitrocellulose paper becomes reactive after treating with aminobenzyl oxymethyl. After blotting RNA to chemically reactive paper, they are hybridized to radiolabelled DNA probe. Autoradiography is then carried out to locate RNA bands that are complementary to the probe (Fig. 13.3(b)).

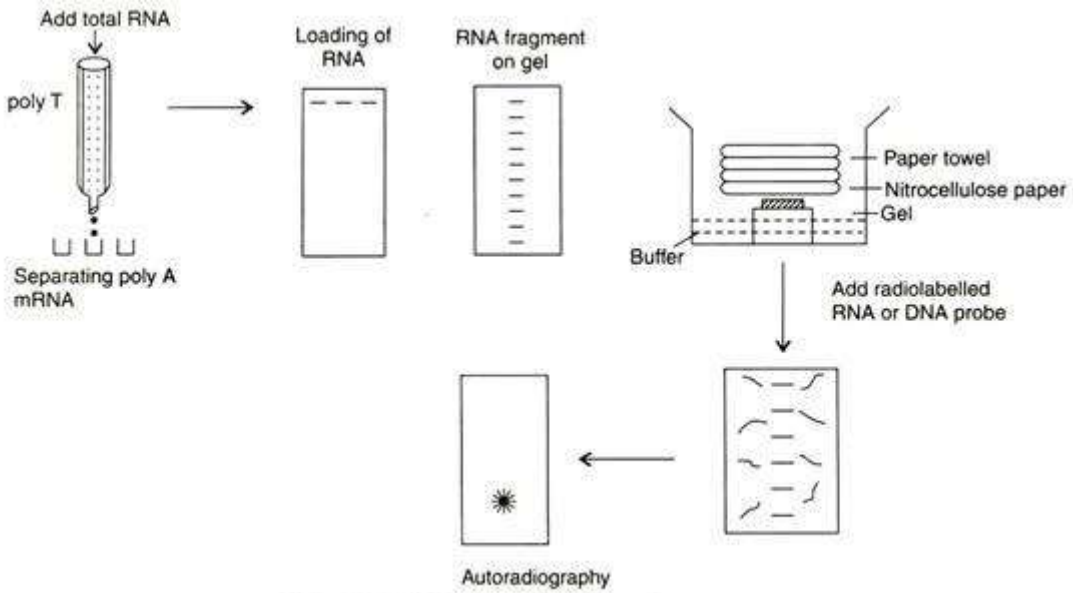


Fig. 13.3 (*b*) Northern hybridization

Significance:

Northern blotting is useful in the identification of a particular gene expression in a tissue or cell type. It is useful in cDNA cloning because the size of a specific mRNA can be compared with the size of cloned cDNA.

III. Western Blotting:

Western blotting is associated with the transfer of proteins from acrylamide gel to nitro-cellulose or nylon membrane. This technique is useful in identification of a particular gene product. Any target protein can be identified by immunological screening method.

Principle:

Western blotting technique is used for identification of particular protein from the mixture of protein. In this method labelled antibody against particular protein is used identify the desired protein, so it is a specific test. Western blotting is also known as immunoblotting because it uses antibodies to detect the protein.

Procedure/Steps:

1. Extraction of protein
2. Gel electrophoresis: SDS PAGE
3. Blotting: electrical or capillary blotting
4. Blocking: BSA
5. Treatment with primary antibody
6. Treatment with secondary antibody(enzyme labelled anti Ab)
7. Treatment with specific substrate; if enzyme is alkaline phosphatase, substrate is p-nitro phenyl phosphate which give colour.

Step I: Extraction of Protein

- Cell lysate is most common sample for western blotting.
- Protein is extracted from cell by mechanical or chemical lysis of cell. This step is also known as tissue preparation.
- To prevent denaturing of protein protease inhibitor is used.
- The concentration of protein is determined by spectroscopy.
- When sufficient amount of protein sample is obtained, it is diluted in loading buffer containing glycerol which helps to sink the sample in well.
- Tracking dye (bromothymol blue) is also added in sample to monitor the movement of proteins.

Step II: Gel electrophoresis

- The sample is loaded in well of SDS-PAGE Sodium dodecyl sulfate- poly-acrylamide gel electrophoresis.
- The proteins are separated on the basis of electric charge, isoelectric point, molecular weight, or combination of these all.
- The small size protein moves faster than large size protein.
- Protein are negatively charged, so they move toward positive (anode) pole as electric current is applied.

Step III: Blotting

- The nitrocellulose membrane is placed on the gel. The separated protein from gel get transferred to nitrocellulose paper by capillary action. This type of blotting is time consuming and may take 1-2 days
- For fast and more efficient transfer of desired protein from the gel to nitrocellulose paper electro-blotting can be used.
- In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

Step IV: Blocking

- Blocking is very important step in western blotting.
- Antibodies are also protein so they are likely to bind the nitrocellulose paper. So before adding the primary antibody the membrane is non-specifically saturated or masked by using casein or Bovine serum albumin (BSA).

Step V: Treatment with Primary Antibody

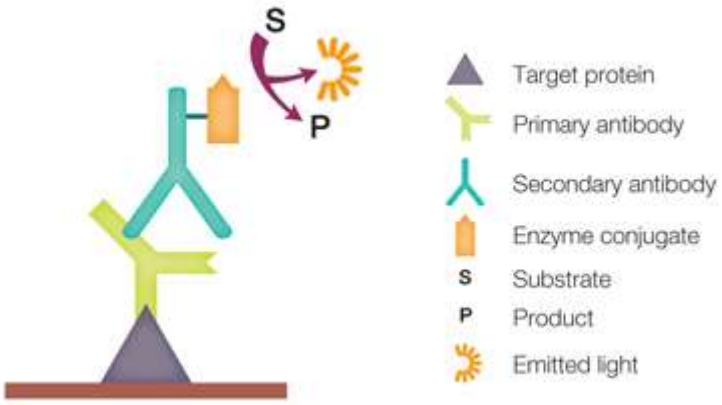
- The primary antibody (1° Ab) is specific to desired protein so it form Ag-Ab complex

Step VI: Treatment with secondary antibody

- The secondary antibody is enzyme labelled. For eg. alkaline phosphatase or Horseradish peroxidase (HRP) is labelled with secondary antibody.
- Secondary antibody (2° Ab) is antibody against primary antibody (anti-antibody) so it can bind with Ag-Ab complex.

Step VII: Treatment with suitable substrate

- To visualize the enzyme action, the reaction mixture is incubated with specific substrate.
- The enzyme convert the substrate to give visible colored product, so band of color can be visualized in the membrane.
- Western blotting is also a quantitative test to determine the amount of protein in sample.



Application:

1. To determine the size and amount of protein in given sample.
2. Disease diagnosis: detects antibody against virus or bacteria in serum.
3. Western blotting technique is the confirmatory test for HIV. It detects anti HIV antibody in patient's serum.
4. Useful to detect defective proteins. For eg Prions disease.
5. Definitive test for Creutzfeldt-Jacob disease, Lyme disease, Hepatitis B and Herpes

Probable Questions:

1. Write down the basic principle of Southern blotting?
2. Describe different steps of Southern Blotting with a suitable diagram.
3. What are the applications of Southern Blotting?
4. Write down the basic principle of Northern blotting?
5. Describe different steps of Northern Blotting with a suitable diagram.
6. What are the applications of Northern Blotting?
7. Write down the basic principle of Western blotting?
8. Describe different steps of Western Blotting with a suitable diagram.
9. What are the applications of Western Blotting?
10. Compare Southern, Northern and Western Blotting techniques.

Suggested Readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7^{th} Edition.
2. Biophysical Chemistry by Upadhyay and Upadhyay.

Unit-XII

Database search tool; Sequence alignment and database searching; Computational tools and biological databases, NCBI, EBL, Sequence similarity tools; Blast and FASTA

Objective: In this unit we will discuss about different search tools which are used for sequence alignment analysis.

Definition of Bioinformatics:

Bioinformatics is currently defined as the study of information content and information flow in biological systems and processes. It serves as the bridge between observations (data) in diverse biologically-related disciplines and the derivations of understanding (information) about how the systems or processes function and subsequently the application (knowledge).

Though Hwa Lim, Father of Bioinformatics, coined the word 'bio/informatique' in 1987, but Temple Smith used the term 'Bioinformatics' in 1991.

In Silico Biology, a new area of Biology, has been developed in recent years because of generation of data in the field of genetics at an unprecedented exponential rate; the management and use of which requires the increasing use of computers and the relevant software.

Computational Biology, another term often used interchangeably with bioinformatics, although the former typically focuses on algorithm development and specific computational methods, while the latter focuses more on hypothesis testing and discovery in the biological domain.

Systems Biology, another area of research, emerged due to availability of enormous amount of molecular data and bioinformatics tools creating unprecedented opportunities to assemble and integrate this data into networks of genes, proteins and bio-chemical pathways.

Bioinformatics involves collection, storage, retrieval and analysis of biological data that has a lot of applications in pharmaceutical, agricultural and food industries, and in molecular genetics research. Biological data are generated from various genome sequencing projects, obtained by different techniques like ONA sequencing (genome and EST), 2D gel electrophoresis, mass spectroscopy (MS, MALDI, LC-MS), protein crystallization, microarrays (e.g., cDNA, oligos, peptide), molecular markers (e.g., RFLP, RAPD, AFLP, SNP). Thus bioinformatics is an interface of biological sciences, mathematics, physical sciences and computer sciences, i.e., the integrated field of biology and information technology.

Major public domain bioinformatics facilities are (Fig. 19.2):

(i) Institutes

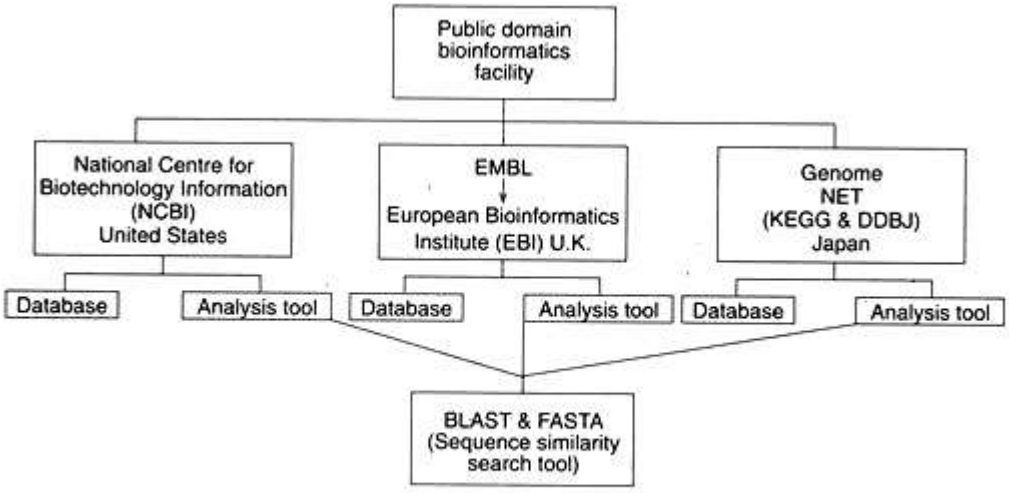


Fig. 19.2: Major publicly available databases and data mining tools

(a) NCBI – National Centre for Biotechnology Information, USA.

(b) EBI – European Bioinformatics Institute, UK.

(c) SIB – Swiss Institute of Bioinformatics, Switzerland.

(d) Genome NET (KEGG & DDBJ), Japan.

ii. Websites:

Some important websites commonly used for bioinformatics are depicted in Table 19.1.

Table 19.1: Some important websites commonly used for bioinformatics (From P. K. Gupta)

Subject	Source	Link
Nucleic acid sequence	Gen Bank	http://www.ncbi.nih.gov:80enterz/query/fcgi?bd-Nucleotide
Genome sequence	SRS at EMBL/FBI	
	Entrez Genome	http://srs.cbiac.uk
	TIGR database	http://www.ncbi.nlm.nin.gov:80/entrez/query.bd=Genome
Protein sequence	GenBank	http//www./tigr.org/tbl/
	SWISS-PORT at ExPASY	http://www.ncbi.nlm.nin.gov:80/entrez/query.fcgi?bd=Protein
	PIR	http://www.expasy.ch/spro/
Protein structure	Protein Data Bank	http://www.ndrf.georgetown.edu
Post translational modifications	RESID	http://www.rcsb.org/pdb/
Biochemical and biophysical information	ENZYME	http://www.ndrf.georgetwon.edu/pirwww/search/textresid.html
	BIND	http://www.expasy.ch/enzyme
Biochemical pathways	Path DB	http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Structure
	KEGG	http://www//ncgr.org/software/pathdb
	WIT	http://www.genome.ad.anl.jp/eegg/
Microarray	Gene Expression Links	http://www.wit.mcs.anl.gov/WIT2/
Other interesting sites	European Bioinformatics	http//industry.ebi.ac.uk/valan/MicroArray
	Institute	http://www.ebi.ac.uk
	DNA Database of Japan	http://www.nig.oc.jp/home.html

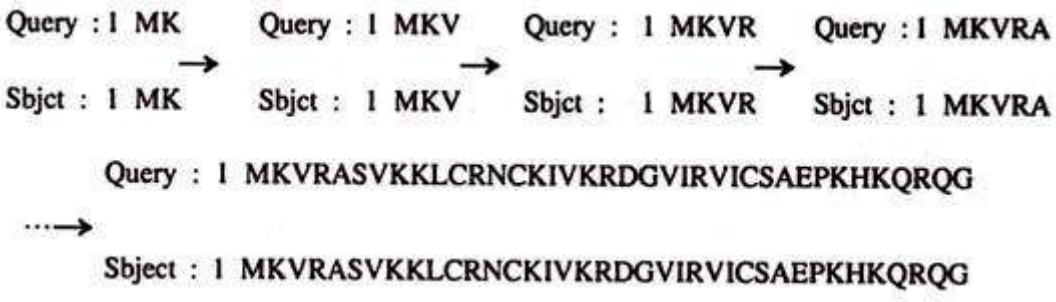
iii. Databases:
Bioinformatics is involved in storing the sequence information in different nucleic acid and protein databases which can be assessed by people all over the world through network technology.

The major protein databases are:
PDB, SWISS-PROT, PROSITE, ExPASy, PIR, PRINTS, BLOCKS, PRODOM, Pfam, Inter Pro.

Nucleic acid databases:
The major nucleic acid databases are: Gen Bank, DDBJ, Ref Seq, dbEST, NDB, CSD, EMBL.

Principles of Sequence Similarity Searches:
The characterization of any new DNA or protein sequence starts with a database search to find out whether homologs of this gene (protein) are available, and in what detail. Clearly, looking for a matching sequence is quite straightforward. Take the first letter of the query sequence, search for its first occurrence in the database, and then check if the second letter of the query is the same in the subject.

If the two letters match, check the third, then the fourth, and continue this comparison to the end of the query. If the match for second letter fails, the search for another occurrence of the first letter will be done, and so on. This will identify all the sequences in the database that are identical to the query sequence (or include it).



Here we looked only for sequences that exactly match the query. To find sequences with the exclusion of the first letter, the same analysis may be conducted with the fragments starting from the second letter of the original query, then from the third one, and so on.

Query 1:1 KVRASVKKLCRNCKIVKRDGVIRVICSAEPKHKQRQG

Query 2:1 VRASVKKLCRNCKIVKRDGVIRVICSAEPKHKQRQG

Query 3:1 RASVKKLCRNCKIVKRDGVIRVICSAEPKHKQRQG

Query 4:1 ASVKKLCRNCKIVKRDGVIRVICSAEPKHKQRQG

These searches, at higher scale, become time-consuming. Finding close relatives would lead to additional conceptual and technical problems. Next, assume that sequences that are 99% identical are definitely homologous. Then, what is the threshold to consider sequences not to be homologous:50% identity, 33%, or perhaps 25% ? The example of two lysozymes shows that sequences with as low as low as 8% identity may belong to orthologous proteins and perform the same function.

Following the information theory of C E Shannon [The Mathematical Theory of Communication, 1949], we can calculate the information content of nucleic acids and of protein. If we use 2-bits (0 or 1 constitute a bit), we can encode 4 units of information (00, 01, 10, 11) which is sufficient to represent one base position in the DNA or RNA.

However, two bases (4-square) are not sufficient to code for the 20 amino acids that are used to constitute the various protein molecules. If we take three bases (4-cube), it gives us a code space of 64 which is more than the requisite 20. This redundancy leads to many codons for each amino acid, error-correcting codes and third place specialties (such as stop codon: TAA, TAG, TGA).

Another aspect is the execution of the "Central Dogma." This is interesting in that it leads to introduction of noise from such sources as vector sequences, heterologous sequences, rearranged & deleted sequences, repetitive element contamination, frame shift errors and sequencing errors or natural polymorphism.

As a matter of fact, all the four nucleotides, A, T, C, and G, are found in the database with approximately the same frequencies and have roughly the same probability of mutating one into another. As a result, DNA-DNA comparisons are largely based on simple text matching, which makes them fairly slow and not particularly sensitive, although a variety of heuristics have been devised to overcome this.

In Contrast, Amino Acid Sequence Comparisons have Several Distinct Advantages, which, at least Potentially, Lead to a Much Greater Sensitivity:

(i) There are 20 amino acids but only four bases. Hence, an amino acid match carries with it > 4 bits of information as opposed to only two bits for a nucleotide match. Thus, statistical significance can be established for much shorter sequences in protein comparisons than in nucleotide comparisons,

(ii) There is redundancy of the genetic code. Almost one-third of the bases in coding regions are under a weak (if any) selective pressure and represent noise, which adversely affects the sensitivity of the searches,

(iii) Nucleotide sequence databases are much larger than protein databases because of the vast amounts of non-coding sequences coming out of eukaryotic genome projects, and this further lowers the search sensitivity,

(iv) Probably most importantly, unlike in nucleotide sequence, the likelihoods of different amino acid substitutions occurring during evolution are substantially different, and taking this into account greatly improves the performance of database search methods. Given all these advantages, comparisons of any coding sequences are typically carried out at the level of protein sequences ; even when the goal is to produce a DNA- DNA alignment (e.g. for analysis of substitutions in silent codon positions), it is usually first done with protein sequences, which are then replaced by the corresponding coding sequences. Direct nucleotide sequence comparison is indispensable only when non-coding regions are analysed.

The laboratory-based as well as research-based sequencing and other types of information relating to the nucleic acids and the proteins are collected as bioinformatics databases in two broad categories: central repository (such as NCBI for nucleotide sequences, Swiss-Prot and PDB for protein sequences, and the smaller ones like Flybase, MGD for mouse genome and RGD for rat genome etc) and combined/secondary databases (such as KEGG for pathway and genome, prosite for annotated protein etc.). The databases are of the most sophisticated type in the computer world and hence require organizational as well as voluntary support for maintenance and upkeep. In fact, the databases are not mere collection of sequences. For example, the PDB (Protein Data Bank) is the single largest worldwide repository for three-dimensional structures of large biological molecules and as early September 2006, it stores 38620 structures.

Thus it houses the sequence, atomic coordinates, derived geometric data, secondary structure content as well as annotations about protein literature references. The PDB was established with 7 structures in 1971 and in 1998, the Research Collaboratory for Structural Bioinformatics (RCSB) was assigned to manage its affairs at Brookhaven National Laboratory.

Substitution Scores and Substitution Matrices:

The fact that each of the 20 standard protein amino acids has its own unique properties means that the likelihood of the substitution of each particular residue for another residue during evolution should be different. Generally, the more similar the physico-chemical properties of two residues, the greater is the chance that the substitution will not have an adverse effect on the protein's function and, accordingly, on the organism's fitness.

Hence, in sequence comparisons, such a substitution should be penalized less than a replacement of amino acid residue with one that has dramatically different properties. This is an oversimplification, because the effect of a substitution depends on the structural and functional environment where it occurs. But, in general, we do not have a priori knowledge of the location of a particular residue in the protein structural and functional environment where it occurs, and even with such knowledge, incorporating it in a database search algorithm is an extremely complex task.

Thus, a generalized measure of the likelihood of amino acid substitutions is applied so that each substitution is given an appropriate value or score (weight) to be used in sequence comparisons. The score for a substitution between amino acids i and j can be expressed by the following intuitively plausible formula, which shows how likely is a particular substitution, given the frequencies of each the two residues in the analysed database:

$$S_{ij} = K \ln (q_{ij}/p_i p_j) \quad (I)$$

where K is a coefficient, q_{ij} is the observed frequency of the given substitution, and p_i, p_j are the background frequencies of the respective residues. Obviously, here the product $p_i p_j$ is the expected frequency of the substitution and, if $q_{ij} = p_i\, p_j$ ($S_{ij} = 0$), the substitution occurs just as often as expected. In practice, the scores used are scaled such that the expected score for aligning a random pair of amino acid sequences is negative.

There are two fundamental ways to design a substitution score matrix, i.e. a triangular table containing 210 numerical score values for each pair of amino acids, including identities (diagonal elements of the matrix). As in many other situations in computational biology, the first approach works abolition, whereas the second one is empirical.

One ab initio approach calculates the score as the number of nucleotide substitutions that are required to transform a codon for one amino acid in a pair into a codon for the other. In this

case, the matrix is obviously unique (as long as alternative genetic codes are not considered) and contains only four values, 0, 1,2, or 3. Accordingly, this is a very coarse grain matrix that is unlikely to work well. The other ab initio approach assigns scores on the basis of similarities and differences in the physico-chemical properties of amino acids.

Under this approach, the number of possible matrices is infinite, and they may have as fine a granularity as desirable, but a degree of arbitrariness is inevitable because our understanding of protein physics is insufficient to make informed decisions on what set of properties "correctly" reflects the relationships between amino acids. Empirical approaches, which came first, attempt to derive the characteristic frequencies of different amino acid substitutions from actual alignments of homologous protein families. In other words, these approaches strive to determine the actual likelihood of each substitution occurring during evolution. Obviously, the outcome of such efforts critically depends on the quantity and quality of the available alignments, and even now, any alignment database is far from being complete or perfectly correct.

Furthermore, simple counting of different types of substitutions will not suffice if alignments of distantly related proteins are included because, in many cases, multiple substitutions might have occurred in the same position, Ideally, one should construct the phylogenetic tree for each family, infer the ancestral sequence for each internal node, and then count the substitutions exactly. This is not practicable in most cases, and various shortcuts need to be taken.

Several solutions to these problems have been proposed, each resulting in a different set of substitution scores. The first substitution matrix, constructed by Dayhoff and Eck (1968), was based on an alignment of closely related proteins, so that the ancestral sequence could be deduced and all the amino acid replacements could be considered occurring just once.

This model was then extrapolated to account for more distant relationships, which resulted in the PAM series of substitution matrices. PAM (Accepted Point Mutaion) is a unit of evolutionary divergence of protein sequences, corresponding to one amino acid change per 100 residues. Thus, for example, the PAM30 matrix is supposed to apply to proteins that differ, on average, by 0.3 change per aligned residue, whereas PAM250 should reflect evolution of sequences with an average of 2.5 substitution per position.

Accordingly, the former matrix should be employed for constructing alignments of closely related sequences, whereas the latter is useful in database searches aimed at detection of distant relationships. Using an approach similar to that of Dayhoff, combined with rapid algorithms for protein sequence clustering and alignment, Jones, Taylor, and Thornton produced the series of the so-called JTT matrices, which are essentially and update of the PAMS. The PAM and JTT matrices, however, have limitations arising out of the fact that they have been derived from alignments of closely related sequences and extrapolated to distantly related ones. This extrapolation may not be fully valid because the underlying evolutionary model might not be adequate, and the trends that determine sequence divergence of closely related sequences might not apply to the evolution at larger distances. In 1992, Steven and Jorja Henikoff developed a series of substitution matrices using conserved ungapped alignments of related proteins from the BLOCKS database. The use of these alignments offered three important advantages over the alignments used for constructing the PAM matrices.

First, the BLOCKS collection obviously included a much larger number and, more importantly, a much greater diversity of protein families than the collection that was available to Dayhoff and coworkers in the 1970's. Second, coming from rather distantly related proteins, BLOCKS alignments better reflected the amino acid changes that occur over large phylogenetic distances

and thus produced substitution scores that represented sequence divergence in distant homologs directly, rather than through extrapolation.
Third, in these distantly related proteins, BLOCKS included only the most confidently aligned regions, which are likely to best represent the prevailing evolutionary trends. These substitution matrices, named the BLOSUM (= BLOCKS Substitution Matrix) series, were tailored to particular evolutionary distances by ignoring the sequences that had more than a certain percent identity. In the BLOSUM62 matrix, for example, the substitution scores were derived from the alignments of sequences that had no more than 62% identity, the substitution scores of the BLOSUM45 matrix were calculated from the alignments that contained sequences with no more than 45% identity.
Accordingly, BLOSUM matrices with high numbers, such as BLOSUMSO, are best suited for comparisons of closely related sequences (it is also advisable to use BLOSUMSO for database searches with short sequences), whereas low-number BLOSUM matrices, such as BLOSUM45, are better for distant relationships. In addition to the general purpose PAM, JTT, and BLOSUM Series, some specialized substitution matrices were developed, for example, for integral membrane proteins, but they never achieved comparable recognition.
Several early studies found the PAM matrices based on empirical data consistently resulted in greater search sensitivity than any of the ab initio matrices. An extensive empirical comparison showed that: (i) BLOSUM matrices consistently outperformed PAMs in BLAST searches and (ii) on average, BLOSUM62 performed best in the series ; this ; this matrix is currently used as the default in most sequence database searches. It is remarkable that, so far, empirical matrices have consistently outperformed those based on theory, either physico-chemical or evolutionary. This perhaps points out that we do not yet have an adequate theory to describe protein evolution.

Statistics of Protein Sequence Comparison:
Let us consider the same protein sequence (E. coli RpsJ) as above

Query ” 1 MKVRASVKKLCRNCKIVKRDGVIRVICSAEPKHKQRQG 38

and check how many times segments of this sequence of different lengths are found in the database (we chose fragments starting from the second position in the sequence because nearly every protein in the database starts with a methionine). Not unexpectedly, we find that the larger the fragment, the smaller the number of exact matches in the database.
With the decrease in the number of database hits, the likelihood that these hits are biologically relevant, i.e. belong to homologs of the query protein, increases. Thus, 13 of the 23 occurrences of the string KVRASV and all 8 occurrences of the string KVRASVK are from RpsJ orthologs.
The number of occurrences of a given string in the database can be roughly estimated as follows. The probability of matching one amino acid residue is 1/20 (assuming equal frequencies of all 20 amino acids in the database ; this not being the case, the probability is slightly greater). The probability of matching two residues in a row is then $(1/20)^2$, and the probability of matching n residues is $(1/20)^n$. Given that the protein database currently contains $N \sim 2 \infty 10^8$ letters, one should expect a string of n letters to match approximately $N \infty (1/20)^n$ times.
Searching for perfect matches is the simplest but insufficient form of sequence database search. However, it is important as one of the basic steps in currently used search algorithms.

Further, the goal of a search is to find homologs, including distant homologs where only a small fraction of the amino acid residues are identical or even similar. Even in close homologs, a region of high similarity is usually flanked by dissimilar regions like in the following alignment of E. coli RpmJ with its ortholog from Vibrio cholerae.

```
E. coli RpmJ :  1  MKVRASVKKLCR---NCKIVKRDGVIRVICSAEPKHKQRQG
                   MKV +S+K      +C+IVKR G + VIC + P + K Q
Vibrio VC0879 :  1  MKVLSSLKSAKNRHPDCQIVKRRGRLYVICKSNPRFKAVQR
```

In this example, the region of highest similarity is in the middle of the alignment, but including the less conserved regions on both sides improves the overall score.
Further along the alignment, the similarity almost disappears so that inclusion of additional letters into the alignment would not increase the overall score or would even decrease it. Such fragments of the alignment of two sequences whose similarity score cannot be improved by adding or trimming any letters, are referred to as high-scoring segment pairs (HSPs). For this approach to work, the expectation of the score for random sequences must be negative, and the scoring matrices used in database searches are scaled accordingly.
So, instead of looking for perfect matches, sequence comparisons programs actually search for HSPs. Once a set of HSPs is found, different methods, such as Smith-Waterman, FASTA, or BLAST, deal with them in different fashions.
However, the principal issue that any database search method needs to address is identifying those HSPs that are unlikely to occur by chance and, by inference, are likely to belong to homologs and to be biologically relevant. This problem has been solved by Samuel Karlin and Stephen Altschul, who showed that maximal HSP scores follow the extreme value distribution. Accordingly, if the lengths of the query sequence (m) and the database (n) are sufficiently high, the expected number of HSPs with a score of at least S is given by the formula

$$E = Kmn2^{-\lambda s} \text{ (II)}$$

Here, S is the so-called raw score calculated under a given scoring system, and K and λ are natural scaling parameters for the search space size and the scoring system, respectively. Normalizing the score according to the formula:

$$S' = (\lambda S - \ln K)/\ln 2 \text{ (III)}$$

gives the bi score, which has a standard unit accepted in information theory and computer science. Then,

$$E = mn2^{-S'} \text{ (IV)}$$

and, since it can be shown that the number of random HSPs with score _ S' is described by Poisson distribution, the probability of finding at least one HSP with bit score _ S' is

$$P = 1 - e^{-E} \text{ (V)}$$

Equation (V) links two commonly used measures of sequence similarity, the probability (P-value) and expectation (E-value). For example, if the score S is such that three HSPs with this score (or greater) are expected to be found by chance, the probability of finding at least one such HSP is $(1 - e^{-3})$, ~ 0.95.

By definition, P-values vary from 0 to 1, whereas E-values can be much greater than 1. The BLAST programs report E- values, rather than P-values, because E-values of, for example, 5 and 10 are much easier to comprehend than P-values of 0.993 and 0.99995. However, for E < 0.01, P-value and E-value are nearly identical.

The product mn defines the search space, a critically important parameter of any database search. Equations (II) and (IV) codify the intuitively obvious notion that the larger the search space, the higher the expectation of finding an HSP with a score greater than any given value. There are two corollaries of this that might take some more time in getting used to: (i) the same HSP may come out statistically significant in a small database and not significant in a large database ; with the natural growth of the database, any given alignment becomes less and less significant (but by no means less important because of that) and (ii) the same HSP may be statistically significant in a small protein (used as a query) and not significant in a large protein. Clearly, one can easily decrease the E-value and the P-value associated with the alignment of the given two sequences by lowering n in equation (II), i.e. by searching a smaller database. However, the resulting increase in significance is false, although such a trick can be useful for detecting initial hints of subtle relationships that should be subsequently verified using other approaches.

It is the experience of the author that the simple notion of E (P)-value is often misunderstood and interpreted as if these values applied just to a single pairwise comparison (i.e., if an E-value of 0.001 for an HSP with score S is reported, then, in a database of just a few thousand sequences, one expects to find a score > S by chance).

It is critical to realize that the size of the search space is already factored in these E-values, and the reported value corresponds to the database size at the time of search (thus, it is certainly necessary to indicate, in all reports of sequence analysis, which database was searched, and desirably, also on what exact date).

The Karlin-Altschul statistics has been rigorously proved to apply only to sequence alignments that do not contain gaps, whereas statistical theory for the more realistic gapped alignments remains an open problem. However, extensive computer simulations have shown that these alignments also follow the extreme value distribution to a high precision ; therefore, at least for all practical purposes, the same statistical formalism is applicable.

Sequence Alignment and Similarity Search:

The Basic Alignment Concepts and Principal Algorithms:

The similarity searches air at identifying the homologs of the given query protein (or nucleotide) sequences in the database. In principle, the only way to identify homologs is by aligning the query sequence against all the sequences in the database (some important heuristics that allow an algorithm to skip sequences that are obviously unrelated to the query are discussed below), sorting these hits based on the degree of similarity, and assessing their statistical significance that is likely to be indicative of homology. Let’s briefly discuss alignment methods first.

It is important to make a distinction between a global (i.e. full-length) alignment and a local alignment, which includes only parts of the analysed sequences (subsequences). Although, in theory, a global alignment is best for describing relationships between sequences, in practice, local alignments are of more general use for two reasons: (i) it is common that only parts of compared proteins are homologous (e.g. they share one conserved domain, whereas other domains are unique), and (ii) often, only a portion of the sequence is conserved enough to carry

a detectable signal, whereas the rest have diverged beyond recognition. Optimal global alignment of two sequences was first implemented in the Needleman-Wunsch algorithm, which employs dynamic programming.

Sequence Database Search Algorithms:

Smith-Waterman:

Any pairwise sequence alignment method in principle can be used for database search in a straightforward manner. All that needs to be done is to construct alignments of the query with each sequence in the database, one by one, rank the results by sequence similarity, and estimate statistical significance.

The classic Smith-Waterman algorithm is a natural choice for such an application, and it has been implemented in several database search programs, the most popular one being SSEARCH written by William Pearson and distributed as part of the FASTA package. It is currently available on numerous servers around the world.

The major problem preventing SSEARCH and other implementations of the Smith-Waterman algorithm from becoming the standard choice for routine database searches is the computational cost, which is orders of magnitude greater than it is for the heuristic FASTA and BLAST methods.

Since extensive comparisons of the performance of these methods in detecting structurally relevant relationships between proteins failed to show a decisive advantage of SSEARCH, the fast heuristic methods dominate the field. Nevertheless, on a case- by-case basis, it is certainly advisable to revert to full Smith-Waterman search when other methods do not reveal a satisfactory picture of homologous relationship for a protein of interest. A modified, much faster version of the Smith-Waterman algorithm has been implemented in the MPSRCH program.

FASTA:

FASTA, introduced in 1988 by William Pearson and David Lipman, was the first database search program that achieved search sensitivity comparable to that of Smith-Waterman but was much faster. FASTA looks for biologically relevant global alignments by first scanning the sequence for short exact matches called “words” ; a word search is extremely fast.

The idea is that almost any pair of homologous sequences is expected to have at least one short word in common. Under this assumption, the great majority of the sequences in the database that do not have common words with the query can be skipped without further examination with a minimal waste of computer time. The sensitivity and speed of the database search with FASTA are inversely related and depend on the “k-tuple” variable, which specifies the word size ; typically, searches are run with k = 3, but, if high sensitivity at the expense of speed is desired, one may switch to k = 2.

Subsequently, Pearson introduced several improvements to the FASTA algorithm, which are implemented in the FASTA3 program.

BLAST:

Basic Local Alignment Search Tool (BLAST) is the most widely used method for sequence similarity search ; it is also the fastest one and the only one that relies on a complete, rigorous statistical theory.

Like FASTA and in contrast to the Smith-Waterman algorithm, BLAST employs the word search heuristics to quickly eliminate irrelevant sequences, which greatly reduces he search time. The program initially searches for a word of a given length W (usually 3 amino acids or 11 nucleotides) that scores at least T When compared to the query using a given substitution matrix.

Word hits are then extended in either direction in an attempt to generate an alignment with a score exceeding exceeding the threshold of S. The W and T parameters dictate the speed and sensitivity of the search, which can thus be varied by the user.

The original version of BLAST (known as BLAST 1.4) produced only ungapped local alignments, for which rigorous statistical theory is available. Although this program performed well for many practical purposes, it repeatedly demonstrated lower sensitivity than the Smith-Waterman algorithm and the FASTA program, at least when run with the default parameters. The new generation of BLAST makes alignments with gaps, for which extensive simulations have demonstrated the same statistical properties as proved for ungapped alignments.

The BLASTX, TBLASTN, and TBLASTX programs are used when either the query or the database or both are uncharacterized sequences and the location of protein-coding regions is not known. These programs translate the nucleotide sequence of the query in all six possible frames and run a protein sequence comparison analogous to that in BLASTP.

A version of gapped BLAST, known as WU-BLAST, with a slightly different statistical model, which, in some cases, may lead to a greater search sensitivity, is supported by Waren Gish at Washington University in St. Louis. Recently, the BLAST suite was supplemented with BLAST2 sequences, a tool for comparing just two nucleotide or protein sequences.

Because of its speed, high selectivity, and flexibility, BLAST is the first choice program in any situation when a sequence similarity search is required, and importantly, this method is used most often as the basis for genome annotation. Therefore, we may consider the practical aspects of BLAST use in some detail. Before that, however, we need to introduce some additional concepts that are critical for protein sequence analysis.

Analysis and Interpretation of BLAST Results:

In spite of the solid statistical foundation, including composition-based statistics, BLAST searches inevitably produce both false positives and false negatives. The main cause for the appearance of false positives, i.e. database hits that have "significant" E- values but, upon more detailed analysis, turn out not to reflect homology, seems to be subtle compositional bias missed by composition-based statistics or low-complexity filtering.

The reason why false negatives are inevitable is, in a sense, more fundamental: in many cases, homologs really have low sequence similarity that is not easily captured in database searches and, even if reported, may not cross the threshold of statistical significance. In an iterative procedure like PSI-BLAST, both the opportunities to detect new and interesting relationships and the pitfalls are further exacerbated.

Beyond the (conceptually) straightforward issues of selectivity and sensitivity, functional assignments based on database search results require careful interpretation if we want to extract the most out of this type of analysis while minimizing the chance of false predictions. Below we consider both the issues of search selectivity and sensitivity and functional interpretation.

No cut-off value is capable of accurately partitioning the database hits for a given query into relevant ones, indicative of homology, and spurious ones. By considering only database hits with very high statistical significance (e.g. $E < 10^{10}$) and applying composition-based statistics,

false positives can be eliminated for the overwhelming majority of queries, but the price to pay is high: numerous homologs, often including those that are most important for functional interpretation, will be missed.
This brief discussion certainly cannot cover all “trade secrets” of sequence analysis. However, the above seems to be sufficient to formulate a few rules of thumb that help a researcher to extract maximal amount of information from database searches while minimizing the likelihood of false “discoveries”.

Probable Questions:

1. Define Bioinformatics.
2. Name major Institutes related to bioinforatics study.
3. What is gap opening penalty and gap extension penalty?
4. How ideal sequence alignment can be achieved?
5. What is FASTA ? State its importance in Bioinformatics .
6. What is BAST? State its importance in Bioinformatics .
7. What is substitution score?
8. What is substitution matrix ?
9. Describe Smith Waterman algorithm.
10. What is Trace back matrix ?

Suggested Readings:

1. Bioinformatics – Principles and Applications – Ghosh and Mullick
2. Bioinformatics by R. Sundaralingam & V Kumarsen – Saras Publication
3. Bioinformatics – Sequence and Genome analysis by David. W. Mount.
4. Introduction to Bioinformatics by Arthur. M. Lesk.

DISCLAIMER: This Self Learning Material (SLM) has been compiled from various authentic books, Journals articles, e-journals and other web sources.

ZOOLOGY

(M. Sc. PROGRAMME)

SEMESTER-IV

ELECTIVE THEORY PAPER:

CELL AND DEVELOPMENTAL BIOLOGY

ZST-412

DIRECTORATE OF OPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Director
Prof. Manas Mohan Adhikary
Directorate of Open and Distance Learning
University of Kalyani

SOFT CORE THEORY PAPER (ZHT- 412)

PARASITE AND DISEASE

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZST - 412 (Parasite and Disease)	I	Primary amoebic meningoencephalitis.	1.0	1	1	5-11
	II	Important Myxozoan genera of fishes - Structure and life history of any *Myxobolus* sp. Important genera of fish parasitic ciliates - *Icthyopthirius* sp.		1	1	12-20
	III	Some common helminthes of freshwater fishes and their life cycle patterns: A) *Proteocephalus* sp. B) *Camallanus* sp.		1	1	21-35
	IV	Structure, Pathobiology prophylaxis and diagnosis of causative agents of filariasis. Parasitic insects: *Cimex lectularius* and *Xenopsylla cheopis*		1	1	36-49

Unit I
Primary amoebic meningoencephalitis

Objective: In this unit you will know about details of Primary amoebic meningoencephalitis

Introduction

Primary amoebic meningoencephalitis (PAM) is a rare and severe disease caused by a single-celled amoeba called *Naegleria fowleri*. *N. fowleri* is known as a "brain eating amoeba" because the microorganism can destroy neurons. It causes inflammation and destruction of the brain and the linings of the brain, stiff neck, fever (38.5°C–41°C), altered mental status, seizures and coma, leading almost always to death.

PAM was first described in South Australia in the 1960s. It has since been identified in many countries throughout the world. Although *Naegleria fowleri* occurs commonly in the environment, it only rarely causes disease. There have been four confirmed cases and one probable case documented in Queensland since the year 2000.

Naegleria fowleri occurs naturally in untreated fresh water, and prefers temperatures between 25°C and 40°C. It can grow in warm, stagnant water bodies such as lakes and hot springs. It can also occur in untreated water piped long distances above ground and in other man-made environments such as poorly maintained swimming pools, wading pools and spas.Infection with *Nigeria fowleri* can occur if water containing the amoebae is pushed up the nose, through activities such as jumping, diving or falling into the water. The amoebae invades humans via intact or disrupted nasal mucosa, crosses the cribriform plate, migrates along the basilar brain from the olfactory bulbs and tracts to the cerebellum, deeply penetrates the cortex to the periventricular system, and incites meningoencephalitis with rapid cerebral oedema, resulting in cerebellar herniation. The olfactory bulbs and orbitofrontal cortices are necrotic and haemorrhagic.The disease cannot be contracted by drinking water or through person to person contact. *Naegleria fowleri* are not found in sea water.

Habit and Habitat

Naegleria feed on yeast, algae and both Gram-negative and Gram-positive bacteria. Food selectivity is observed with findings that filamentous cyanobacteria (e.g., *Anabaena*, *Cylindrospermum*, *Gloeotrichia*, and *Phormidium*) are consumed, while tight threads (*Oscilltoria*) and aggregates (*Aphanizomenon*) are not ingested. Unicellular Chroococcaceae (e.g., *Synechococcus*, *Aphanocapsa*, and *Microcystis*) are excreted after ingestion, indicating that food selection takes place inside food vacuoles. Ingestion depends on the satiation status of the amoebae, as starved amoebae feed at higher rates compared with satiated amoebae. *N. fowleri* can be grown simply on the surface of non-nutrient agar overlaid with living or dead *Enterobacter aerogenes* or *E. coli* or other Gram-negative bacilli. Live bacteria support optimal growth compared with heat-killed bacteria. Under these conditions, the amoebae feed upon the bacteria, and as growth enters stationary phase and the food supply is used up, *N. fowleri* begin to encyst. Cysts, if kept from drying out, will remain viable for months, possibly years.

Life cycle

Naegleria fowleri is a protist pathogen that is extensively dispersed in the environment. *N. fowleri* is the only pathogenic species in this genus. Given the opportunity and access, *N. fowleri* can cause fatal

primary amoebic meningoencephalitis (PAM). Worryingly, mortality rates concomitant with PAM remain substantially high, irrespective of modern improvements in antimicrobial chemotherapy or supportive medical care. Being a free-living amoeba, *N. fowleri* can switch phenotype depending on the environmental conditions (Fig. 1).

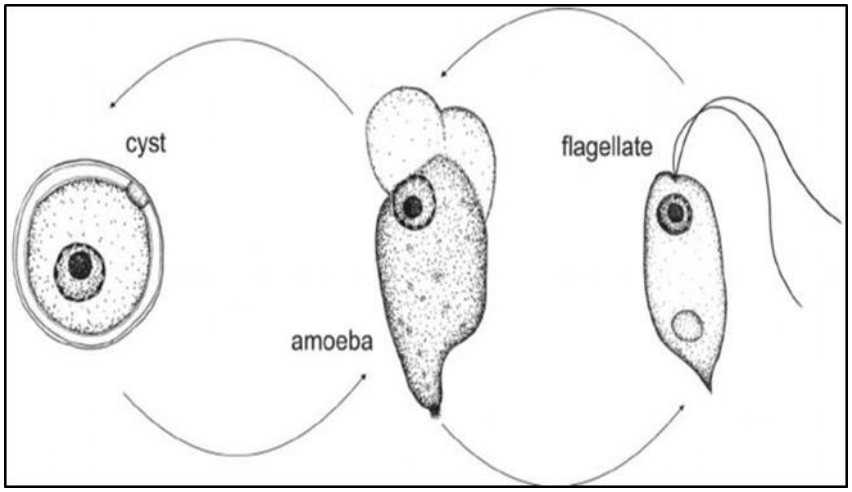

Fig 1: Different phenotypes of *N. fowleri*

Under favourable conditions, it exhibits a reproductively-active trophozoite stage. The trophozoite stage is considered as the infective stage. Under non-nutrient conditions; but presence of water, trophozoites switch to a transient flagellate stage allowing long distance movement, often in pursuit of nourishment. During this stage, *N. fowleri* does not reproduce or form cyst. When the environment is adverse or unfavourable, trophozoites switch into a metabolically inactive or dormant form known as the cyst form.

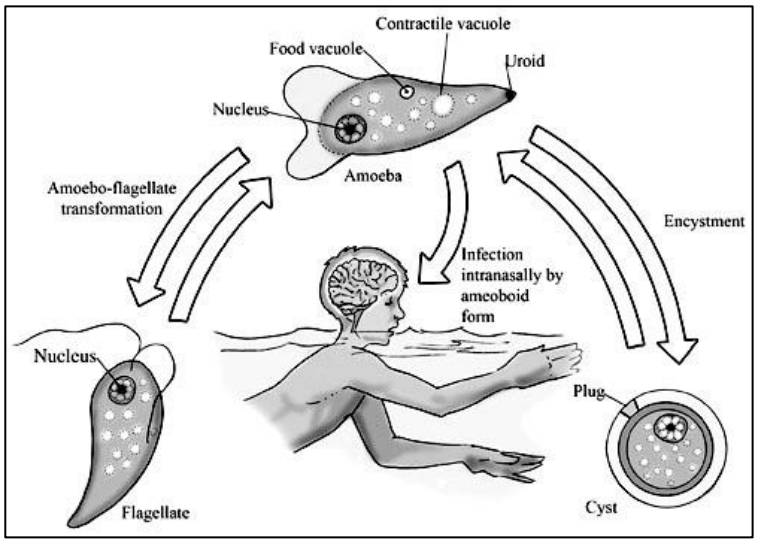

Fig 2: Life cycle of *N. fowleri*

Like the flagellate phase, the cysts are non-feeding, and non-reproductive. Only the trophozoites of N. fowleri can feed, reproduce, and/or become cysts. The parasites enter hosts through the nasal route, travelling via the olfactory neuroepithelial and thus gaining entry to the central nervous system with the production of PAM (Fig. 2).

CNS infection by *N. fowleri* occurs by the amoebae passing through the nasal cavity, penetrating the olfactory neuroepithelium, migrating through the olfactory nerves (Fig. 3) and crossing the cribriform plate until they reach the olfactory bulbs (OBs)

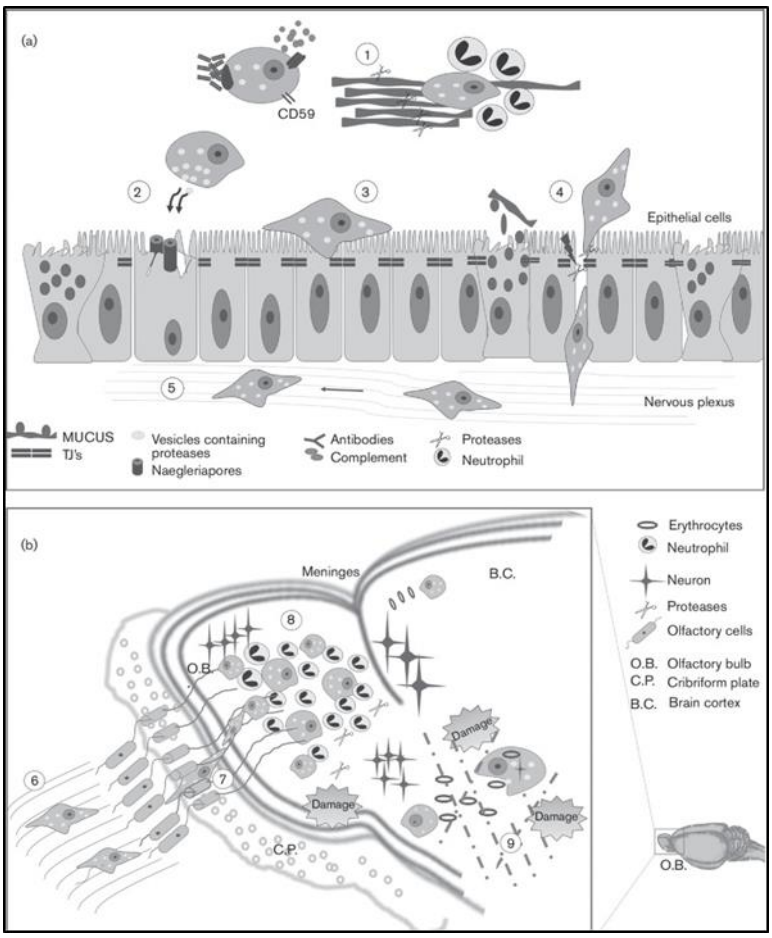

Fig 3: Schematic representation of *N. fowleri* infection.

(a) Initial stages of PAM. (1) Evasion of innate immune response, (2) independent-contact cytotoxicity (naegleriapores), (3) adhesion to epithelial cells, (4) invasion of the neuroepithelium, (5) migration to OBs.

(b) Late stages of PAM. (6) Contact with olfactory phyla, (7) amoeba crossing the cribriform plate, (8). *N fowleri* proliferation and inflammatory reaction in the OBs, (9) tissue damage (haemorrhage, phagocytosis and protease release).

Clinical and laboratory diagnosis

Patients exhibiting CNS symptoms together with a history of swimming or use/exposure to contaminated water for nasal cleansing should be suspected of PAM.

- The computed tomographic scan (CT) reveal involvement of the CNS, around and above the midbrain and the subarachnoid spaces are eliminated on pre-contrast CT. Noticeable augmentation is seen after intravenous contrast medium administration. Whereas the ventricular size is usual, the sulci and adjacent grey matter are also intensely enhanced.
- The definitive diagnosis of PAM involves CSF (Cerebro-Spinal Fluid) findings, i.e., presence of amoebae in the CSF. In the majority of cases, motile trophozoites are observed in CSF by wet mount. Brief centrifugation of the CSF at 5,000 x g for 5 min is helpful to concentrate amoebae.

- In addition to microscopy, immunofluorescence assay (IF), enzyme-linked immunosorbent assay (ELISA), flow cytometry, and PCR-based assays have been developed. Assays should be employed on both CSF and nasal exudates.
- Apart from the presence of amoebae, CSF findings in PAM are comparable to bacterial meningitis. For example, the red blood cell count in CSF increases several fold from 250 cells per mm3 in the early stage to 25,000 cells per mm^3 in the late stage. Similarly, the white blood cell count is raised, with a polymorphonuclear leukocyte predominance, with a range of 300 cells per mm3 to as high as 26,000 cells per mm^3. The CSF pressure is typically raised (300 – 600 mm H2O). The protein concentration can range from 100 mg per 100 mL to 1000 mg per 100 mL, while glucose might be 10 mg per 100 mL or less.
- Endeavours ought to be made to culture the amoebae from the CSF. A few drops of CSF should be transferred to a non-nutrient agar plate seeded with bacteria and amoebae growth should observed daily for up to seven days. Amoebae appear as the trophozoite form within 1 – 2 days. *N. fowleri* can be differentiated from other pathogenic amoebae using enflagellation experiment by mixing one drop of amoebae culture or sedimented CSF and 1 mL of distilled water for 1 – 2 h with periodic observation for the presence of flagellates, however the molecular methods remain the method of choice.

Pathogenesis

In vivo, ex vivo and *in vitro* models have been developed to study molecular mechanisms associated with N. fowleri pathogenesis. *In vivo,* mice are inoculated intranasally with *N. fowleri* that results in high mortality rate. The susceptibility of mice is influenced by weight (mice weighing less than 15 g are more sensitive), and age (younger mice are more sensitive). Following infection, *N. fowleri* are observed in mucous layer of the olfactory epithelium within 8 h post-infection and infected mice exhibit focal inflammation with the presence of *N. fowleri* in the submucosal nerve plexus, olfactory nerves penetrating the cribriform plate, and the olfactory bulb of the brain within 24 h post-infection. Following 96 h, the inflammatory response, primarily in the form of neutrophil polymorphs, is severe in the olfactory bulb and the brain, with tissue damage. Numerous amoebae are seen interspersed with the degenerating neurones, glial processes, and neutrophil polymorphs with major concentrations in the perivascular regions and in the lamina of blood vessels.

When *N. fowleri* are incubated with host cells *in vitro*, host cells show cell shrinkage, cell damage, invasion and destruction via phagocytic processes.

In *ex vivo* model, infection of organotypic slice cultures from rat brain with amoebae is comparable to findings with *in vivo* infection, suggesting its usefulness in the study of *N. fowleri* pathogenesis.

The pathogenicity of *N. fowleri* is discussed below-

N. fowleri is an amphizoic amoeba as it can survive in a free-living state in water, soil, or in the host which can be the human central nervous system (CNS). *N. fowleri* infections have been documented in healthy children and adults following recreational water activities including swimming, diving, and water skiing. *N. fowleri* has been thought to infect the human body by entering the host through the nose when water is splashed or forced into the nasal cavity. Infectivity occurs first through attachment to the nasal mucosa followed by locomotion along the olfactory nerve, through the cribriform plate (which is more porous in children and young adults), and finally reaching the olfactory bulbs within the CNS. Once *N. fowleri* reaches the olfactory bulbs, it elicits a significant immune response through activation of the innate immune system including macrophages and neutrophils. *N. fowleri* enters the

human body in the trophozoite form. Structures on the surface of trophozoites known as food-cups enable the organism to ingest bacteria, fungi, and human tissue. In addition to tissue destruction by the food cup, the pathogenicity of *N.fowleri* is dependent upon the release of cytolytic molecules including, acid hydrolases, phospholipases, neuraminidases, and phospholipopolytic enzymes that play a role in host-cell and nerve destruction. The combination of the pathogenicity of *N.fowleri* and the intense immune response resulting from its presence results in significant nerve damage and subsequent CNS tissue damage, which often result in death.

Prevention

Naegleria fowleri cannot survive in water that is clean, cool and chlorinated. To prevent infection:

- avoid jumping or diving into bodies of warm fresh water or thermal pools
- keep your head above water in spas, thermal pools and warm fresh water bodies
- empty and clean small collapsible wading pools daily
- ensure swimming pools and spas are adequately chlorinated and well maintained
- flush stagnant water from hoses before allowing children to play with hoses or sprinklers
- if you are using unchlorinated water:
    - don't allow water to go up your nose when bathing, showering or washing your face
    - supervise children playing with hoses or sprinklers and teach them not to squirt water up their nose
- potentially contaminated water should not be used for any form of nasal irrigation or nasal lavage including Neti (an Ayurvedic practice of nasal cleansing)

Treatment

- It is important to highlight that an appropriate diagnosis is the key to choosing an appropriate treatment. However, PAM is not commonly confirmed during the early stages of infection, and most people infected with this organism die. Because of the high mortality rate, more effective drugs are urgently needed.
- Few people survive Naegleria infection, even with treatment. Early diagnosis and treatment are crucial for survival.
- The primary treatment for Naegleria infection is an antifungal drug, amphotericin B — usually injected into a vein (intravenously) or into the space around your spinal cord to kill the amoebas.
- An investigational drug called miltefosine (Impavido) is now available for emergency treatment of Naegleria infection. The medicine, when taken with other medications and along with aggressive management of brain swelling, may show promise for improved survival.

Control

Chlorine kills Naegleria fowleri and is the most effective way to disinfect swimming pools and reticulated water supplies. In rural water supplies, chlorine may not reach areas where the amoeba may form colonies. In such circumstances, a process called *chloramination* is more effective to

control *Naegleria fowleri*. Filtration and UV treatment systems may be effective in controlling *Naegleria fowleri*, but specialist advice should be sought.

Signs and Symptoms

The typical symptoms of PAM appear during the first week after infection with *N. fowleri* trophozoites. There are no distinctive clinical features to differentiate PAM from other types of meningitis. Therefore, it is very important that physicians obtain a detailed clinical history of the patients. The earliest symptoms include

- severe headache,
- high fever and neck stiffness,
- anorexia,
- vomiting,
- irritability,
- photophobia and
- Neurological abnormalities, including diplopia, lethargy, seizures and coma.

Cranial nerve palsies may indicate brain oedema. Death occurs between the third and seventh days after symptom onset.

Autopsies of PAM patients have revealed brain inflammation with severe tissue damage throughout the area of invasion, with ulceration of the olfactory mucosa and necrosis of the olfactory nerves. Microscopically, the OBs were almost completely disorganized by fibrin-purulent exudates and by haemorrhaging from necrotic blood vessels, and the adjacent frontal cortex exhibited the invasion of a considerable number of amoebae.

Conclusion

PAM is an acute and fatal disease that has recently become more common in both developed and underdeveloped countries. The number of PAM cases may increase due to global warming, global overpopulation and increased industrial activities. It is urgent that the health community, including medical and diagnostic laboratory technicians, be aware of this disease in order to make timely diagnosis that could save patients' lives. The knowledge of the biology and pathogenesis of *N. fowleri* in the past 50 years could be used to make faster diagnosis and design new drugs against specific targets to eliminate the amoeba and increase the survival of the patients.

Probable questions:

1. Describe the life cycle of *Naegleria fowleri* with diagram
2. Briefly discuss different forms of *Naegleria fowleri* with diagram
3. Describe with diagram penetration and migration of *Naegleria fowleri* through olfactory nerves.
4. Write down the control measures and treatment of *Naegleria fowleri*
5. Describe pathogenicity and role in disease transmission of *Naegleria fowleri.*
6. Describe the morphology of *Naegleria fowleri.*

Suggested reading:

1. https://www.cdc.gov/parasites/naegleria/index.html
2. http://conditions.health.qld.gov.au/HealthCondition/condition/14/165/115/primary-amoebic-meningoencephalitis-pam
3. Roberts, L.S and Janovy, J. (2009). Smith & Robert's Foundation of Parasitology. 8th. Edn. McGraw Hill
4. John, D.T. and W.A. Petri (2006). Markell and Voge's Medical Parasitology. 9th Edn. Elsevier.

Unit II

Important Myxozoan genera of fishes - Structure and life history of any *Myxobolus* sp. Important genera of fish parasitic ciliates - *Icthyopthirius* sp.

Objective: In this unit you will know about important Myxozoan genera of fishes and important genera of fish parasitic ciliates like *Myxobolus* sp. and *Icthyopthirius* sp. respectively; emphasising on their structure and life history.

Introduction

Myxobolus cerebralis is a myxosporean parasite of salmonids (salmon, trout, and their allies) that causes *whirling disease* in farmed salmon and trout and also in wild fish populations. Whirling disease is so called because fish with the disease swim in circles when disturbed or feeding. It was first described from rainbow trout in Germany a century ago, but its range has spread and it has appeared in most of Europe (including Russia), the United States, South Africa and other countries. In the 1980s, it was discovered that *M. cerebralis* needs to infect a tubificid oligochaete (a kind of segmented worm) to complete its life-cycle. The parasite infects its hosts with its cells after piercing them with polar filaments ejected from nematocyst-like capsules.

Whirling disease afflicts juvenile fish (fingerlings and fry) and causes skeletal deformation and neurological damage. Fish "whirl" rather than swim forward, find feeding difficult, and are more vulnerable to predators. The mortality rate is high for fingerlings, up to 90% of infected populations, and those that do survive are deformed by the parasite residing in their cartilage and bone. They act as a reservoir for the parasite, which is released into water following the fish's death. *M. cerebralis* is one of the most economically important myxozoans in fish as well as one of the most pathogenic. It was the first myxosporean whose pathology and symptoms were described scientifically. The parasite is not transmissible to humans.

Systematic position

Phylum: Cnidaria
Subphylum: Myxozoa
Class: Myxosporea
Class: Myxobolus cerebralis
Order: Bivalvulida
Suborder: Platysporina
Family: Myxobolidae
Genus: *Myxobolus*
Species: *Myxobolus cerebralis*

Taxonomy

The taxonomy and naming of both *M. cerebralis* and of myxozoans in general have complicated histories. It was originally thought that this parasite infected fish brains (hence the specific epithet cerebralis), however it quickly became apparent that while it can be found in the nervous system, it primarily infects cartilage and skeletal tissue. Attempts to change the name to *Myxobolus chondrophagus*, which would more accurately describe the organism, failed because of nomenclature rules. Later, it became apparent that organisms previously called *Triactinomyxon dubium* and *T.*

gyrosalmo(class Actinosporea) were in fact triactinomyxon stages of *M. cerebralis*, whose life cycle was expanded to include the triactinomyxon stage. Similarly, other actinosporeans were folded into the life cycles of various myxosporeans.

Today, the myxozoans, previously thought to be multicellular protozoans are considered animals by many scientists, though their status has not officially changed. Recent molecular studies suggest that they are related to Bilateria or Cnidaria, with Cnidaria being closer morphologically because both groups have extrusive filaments, but with Bilateria being somewhat closer in some genetic studies.

Morphology

M. cerebralis has many diverse stages ranging from single cells to relatively large spores, not all of which have been studied in detail.

Triactinomyxon stage

The stages that infect fish, called triactinomyxon spores, are made of a single style that is about 150 micrometers (µm) long and three processes or "tails" that are each about 200 micrometers long (Fig 1). A sporoplasm packet at the end of the style contains 64 germ cells surrounded by a cellular envelope. There are also three polar capsules, each of which contains a coiled polar filament between 170 and 180 µm long. Polar filaments in both this stage and in the myxospore stage (see picture above) rapidly shoot into the body of the host, creating an opening through which the sporoplasm can enter.

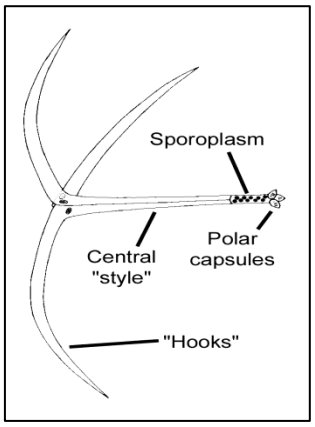

Fig 1: Diagram of the structure of a triactionmyxon stage spore of *Myxobolus cerebralis*

Sporoplasm stage

Upon contact with fish hosts and firing of the polar capsules, the sporoplasm contained within the central style of the triactinomyxon migrates into the epithelium or gut lining. Firstly, this sporoplasm undergoes mitosis to produce more amoeboid cells, which migrate into deeper tissue layers, in order to reach the cerebral cartilage.

Myxosporean stage

Myxospores, which develop from sporogonic cell stages inside fish hosts, are lenticular. Mature spores of *M. cerebralis* are broadly oval, with thick sutural ridges on the valve edges. They measure 7.4-9.7 µm long by 7-10 µm wide and are made of six cells. Spores are covered with a mucoid-like envelope. There are two polar capsules at the anterior end, each with a filament twisted into five or six coils. Twopolar capsules merge to form a binucleate sporoplasm, and two form protective valves. During development each polar capsule lies within a polar cell that also contains a nucleus, and nuclei of the two valvogenic cells may be seen lying adjacent to the inner surface of each valve. The

sporoplasm contains two nuclei (presumably haploid), numerous ribosomes, mitochondria, and other typical organelles.

Myxospores are infective to oligochaetes, and are found among the remains of digested fish cartilage. They are often difficult to distinguish from related species because of morphological similarities across genera. Though *M. cerebralis* is the only myxosporean ever found in salmonid cartilage, other visually similar species may be present in the skin, nervous system, or muscle.

Life cycle

Myxobolus cerebralis has a two-host life cycle that involves the salmonid fish and an alternate host, the bottom-dwelling *tubifex* worm (*Tubifex tubifex*) (Fig 2).

First, myxospores are ingested by tubificid worms. In the gut lumen of the worm, the spores extrude their polar capsules and attach to the gut epithelium by polar filaments. The shell valves then open along the suture line and the binucleate germ cell penetrates between the intestinal epithelial cells of the worm. This cell multiplies, producing many amoeboid cells by an asexual cell fission process called merogony. As a result of the multiplication process, the intercellular space of the epithelial cells in more than 10 neighbouring worm segments may become infected.

Around 60–90 days post-infection, sexual cell stages of the parasite undergo sporogenesis, and develop into pansporocysts, each of which contains eight triactinomyxon-stage spores. These spores are released from the oligochaete anus into the water. Alternatively, a fish can become infected by eating an infected oligochaete. Infected tubificids can release triactinomyxons for at least 1 year.

The triactinomyxon spores swim through the water to infect a salmonid through the skin. Penetration of the fish by these spores takes only a few seconds. Within five minutes, a sac of germ cells called a sporoplasm has entered the fish epidermis, and within a few hours, the sporoplasm splits into individual cells that will spread through the fish.

Within the fish, there are both intracellular and extracellular stages that reproduce in its cartilage by asexual endogeny, meaning that new cells grow from within old cells. The final stage within fish is the myxospore, which is formed by sporogony. They are released into the environment when the fish decomposes or is eaten. Some recent research indicates that some fish may expel viable myxospores while still alive.

Myxospores are extremely tough: "it was shown that *Myxobolus cerebralis* spores can tolerate freezing at -20°C for at least 3 months, aging in mud at 13°C for at least 5 months, and passage through the guts of northern pike *Esox lucius* or mallards *Anas platyrhynchos* without loss of infectivity" to worms. Triactinomyxons are much shorter lived, surviving 34 days or less, depending on temperature.

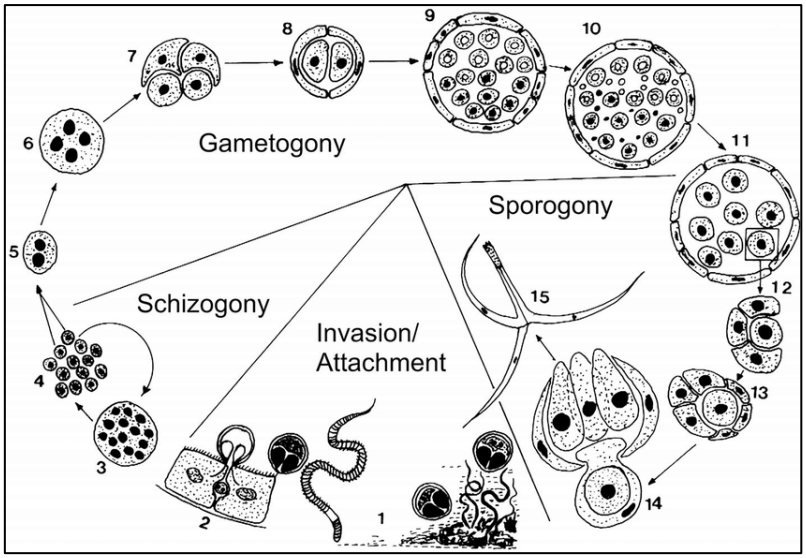

Fig 2: *Myxobolus cerebralis* life cycle: development of myxospore in oligocheate host *Tubifex tubifex*.

Stage 1: ingestion of M. cerebralis by T. tubifex.

Stage 2: extrusion of the polar filaments and anchorage of M. cerebralis spore into gut epithelium. After shell valves open, binucleate sporoplasm escapes and penetrates between epithelial cells.

Stage 3: interepithehal schizogonic multiplication of binucleate sporoplasm.

Stage 4: uninucleate 1-cell stages.

Stage 5: plasmogamy of 2 uninucleate cells to produce one binucleate cell-stage.

Stage 6: mitotic division of both nuclei to produce 4-nuclei stage.

Stage 7: formation of 4-cell stage by plasmotomy; 2 cells begin to envelop the other 2 cells.

Stage 8: formation of early pansporocyst with 2 somatic and 2 generative cells.

Stage 9: following 3 mitotic divisions of both generative cells and 2 mitotic divisions of the somatic cells, 16 gametocytes (8α and 8β) enveloped by 8 somatic cells are formed.

Stage 10: following meiotic division of the 16 diploid gametocytes, 16 haploid gametocytes and 16 polar bodies result.

Stage 11: production of 8 zygotes after copulation of each pair of α-and β-gametes.

Stage 12: sporoblast formation after 2 mitotic divisions of zygote, 3 pyramidally arranged cells and one inner cell are formed.

Stage 13: following mitotic division of the 3 peripheral cells, 3 capsulogenic and 3 valvogenic cells are produced.

Stage 14: the valvogenic cells extend around the capsulogenic cells, while internal cleavage of the developing sporoplasm cell produces one generative cell enveloped by one somatic cell. The sporoplasm remains naked in the pansporocyst until reaching the final number of germs through repeating mitotic divisions.

Stage 15: inflated mature triactinomyxon spore.

Pathogenesis

The main pathogenic effects of this disease are damage to cartilage in the axial skeleton of young fish, consequent interference with function of adjacent neural structures, and subsequent granuloma formation in healing of the lesions. Invasion of the cartilaginous capsule of the auditory-equilibrium organ behind the eye interferes with coordinated swimming. Thus, when an infected fish is disturbed or tries to feed, it begins to whirl frantically, as if chasing its tail. It may become so exhausted by this futile activity that it sinks to the bottom and lies on its side until it regains strength. Predation most likely occurs at this stage. Often the spine cartilage is invaded, especially posterior to the 26th vertebra. Function of sympathetic nerves controlling melanocytes is impaired, and an infected fish's

posterior part becomes very dark, producing the “black tail.” If the fish survives, granulomatoustissue infiltration of the skeleton may producepermanent deformities: misshapen head, permanently open or twisted lower jaw, or severe spinal curvature

(a) (b)

Fig 3: Axial skeleton deformities in living rainbow trout that have recovered from whirling disease (*Myxobolus cerebralis*)

(*a*) Note bulging eyes, shortened operculum, and both dorsoventral and lateral curvature of the spinal column (lordosis and scoliosis).

(*b*) Note gaping, underslung jaw and grotesque cranial granuloma.

• Fish parasitic ciliates - *Ichthyophthirius multifiliis*

Introduction

Ichthyophthirius multifiliis is a large, ciliated protozoan that causes "Ich" or "white spot disease." This disease is a major problem to freshwater aquarists and commercial fish producers worldwide. All species of freshwater fish are considered susceptible, and the parasite has been found in all areas of the world in both cultured and wild fish. These large parasites cause the characteristic white spots that are often seen on the skin and fins of infected fish. The disease is highly contagious and spreads rapidly from one fish to another without the need for additional hosts (direct life cycle). Although often considered a “warm water” disease, outbreaks often occur when water temperatures are changing, especially in the spring when water temperatures are increasing. The disease is particularly severe when fish are crowded. While many protozoans reproduce by simple division (one parasite “splits” into two), a single “Ich” organism can multiply into hundreds of new parasites in one generation, making early detection and treatment of this parasite crucial. The organism is unusual in that it is an obligate parasite, which means that it cannot survive unless live fish are present. “Ich” is capable of causing massive mortality within a short period of time. An outbreak of “Ich”is a true emergency situation and requires immediate treatment; if left untreated, this disease may result in 100% mortality.

Parasite morphology

The parasite forms three developmental stages: trophonts, tomonts and theronts (Fig 4). ***Trophonts*** variable in size (up to 1mm), horseshoe-shaped macronucleus encircling single micronucleus; subapical vestibulum with weakly developed buccal ciliature ***tomonts*** encysted on substrate, repeatedly divides to form numerous small tomites which break through cyst wall to become

theronts(25-70 x 15-22µm) covered with 36-48 meridional (longitudinal) kineties (ciliary rows) converging around the pre- and post-oral sutures. Ellipsoidal macronucleus and subspherical micronucleus.

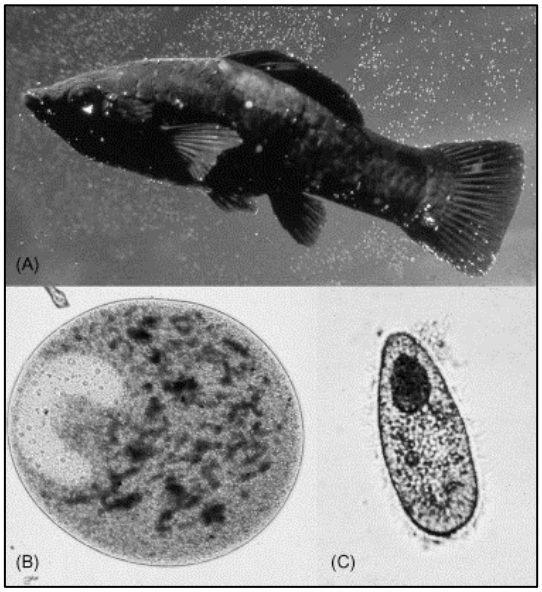

Fig 4: (A) Black molly with a few variably sized white spots caused by *Ichthyophthirius* cysts and the milky nature of the mucous coat that is seen in any condition where irritation causes excess mucus production. (B) *Ichthyophthirius* tomont with its characteristic horseshoe shaped nucleus (C) The much smaller free-swimming infective theront of *Ichthyophthirius*.

Host range

Infections have been detected in numerous species of aquarium and wild freshwater fish throughout the world. There is some conjecture about the existence of different parasite races, which may have different temperature tolerances, being adapted to hosts with specific temperature preferences, or they may be geographic races varying in virulence in introduced and/or endemic fish species.

Site of infection

Trophonts infect the epidermis, cornea and gill filaments.

Life Cycle

Although *Ichthyophthirius multifiliis* has a direct life cycle, it is fairly complex and has three distinct life stages:

1) The on-fish, feeding trophont;
2) The environmental, reproducing tomont; and
3) The infective, fish-seeking theront (Fig 5).

The trophont invades and encysts between the thin outer layers of the fish host's skin and gills in order to feed on those tissues. Because of the covering by this epithelial tissue and mucus, the trophont stage is protected from chemical treatment. Once the trophont is mature, it stops feeding, leaves the fish, and becomes a tomont. The tomont quickly secretes a gelatinous-walled outer cyst that allows it to stick to surfaces in the environment. The tomont begins to divide quickly, forming hundreds of new "daughter" parasites (tomites) within a single cyst. This can occur in a day or less at

warmer water temperatures. The gelatinous wall of the tomont cyst protects it and the daughter tomites from chemical treatment.

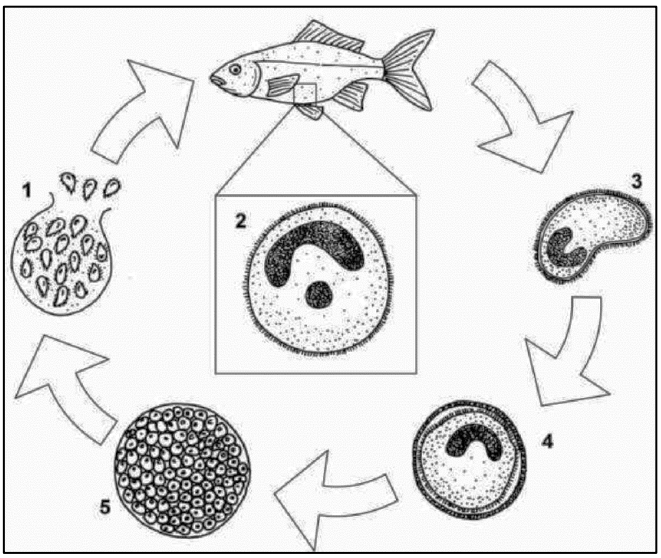

Fig 5: Life-cycle of *Ichthyophthirius multifiliis*. 1) Infective theronts released from cyst. 2) Parasitic trophont stage. 3) Exiting tomont. 4) Cyst. 5) Dividing tomites within cyst.

The tomites begin to develop and become theronts within the tomont cyst. Following a period of days (warm water temperatures) or weeks (cool water temperatures), the theronts bore out of the tomont cyst and become free-swimming, infective parasites in search of a fish host. These infective theronts must find a live fish to complete the parasite's life cycle. This free-swimming phase is unprotected and, therefore, highly susceptible to chemicals. Treatment protocols should be designed to target this theront stage.

Pathogenesis

Theronts use an elevated pointed ridge (perforatorium) to penetrate host tissues and they discharge their pellicular mucocysts to form a stick envelope glued to the host's epithelium. Within minutes, the parasites penetrate deeper into epithelial or epidermal tissues where they feed and grow (increasing their volume up to 3,000 times). The trophonts form greyish pustules in the skin of their hosts where they feed by ingesting host cell debris. Infected fish produce excess mucus to combat the irritation but many epidermal cells are destroyed and are sloughed. Heavy infections of the gill filaments interfere with gas exchange and may prove fatal. Lesions containing engorging trophonts appear as visible white spots covering infected fish. Fish surviving infection exhibit some protective immunity against subsequent infections.

Mode of transmission

Engorged trophonts are liberated from ruptured pustules into the water column where they settle on convenient substrates or on the bottom. They form a gelatinous cyst and undergo a series of divisions producing from 250 to 2,000 tomites which are subsequently released and actively search for new hosts. The number, size and duration of the life-cycle stages depends prevailing environmental condition, particularly temperature (no development occurs below 2°C or above 30°C). The whole life-cycle may be completed in as little as 3-8 days at 23-24°C, but it progressively takes longer at lower temperatures (up to 3 months at 4-5°C).

Disease Signs

The classic sign of an “Ich” infection is the presence of small white spots on the skin or fins (Fig 6). These spots are caused as the adult parasite (trophont) penetrates and creates a space in the outer layers of the fish’s body surfaces (epithelium) in order to feed on the fish and move around. These lesions look like small white dots, blisters, or salt grains on the skin or fins of the fish. The white spots may not be as obvious on fish that are white or pale in color, or if the infection is limited to the gills. By the time the white spots are visible to the naked eye, the infected fish is very sick. Prior to the appearance of white spots, fish may have shown signs of irritation, flashing, weakness, loss of appetite, and decreased activity. A well-trained aquaculturist or aquarist will detect these changes before the fish’s condition worsens and mortalities occur. If the parasite is only present in the gills, white spots may not be seen at all but fish will die in large numbers. In these fish, gills will often be pale and very swollen. White spots should never be used as the only means of diagnosis because other diseases may have a similar appearance. Gill and skin biopsies should be collected and examined with a light microscope when the first signs of illness are observed. If even a single “Ich” parasite is seen, fish should be medicated immediately because as the infection advances fish may not survive, even with treatment.

Fig 6: “Ich” trophont—feeding stage found on fish.

Prevention

Prophylactic treatments with saline baths or dilute copper are used on incoming fish during quarantine in some facilities. The immunocompromising impact of copper treatments is increasingly well documented. Maintenance of excellent water quality and minimization of stress are thought to reduce the likelihood of a clinical outbreak. Adequate water changes and cleaning of substrates are thought to help prevent accumulation of high numbers of infective tomonts.

Control

Heavy filtration with diatomaceous earth or membrane filters will reduce the number of circulating theronts. Transferring fish to clean aquaria every day for 7 days will limit the infection by keeping one step ahead of theront reinfestation. Removal of theronts from the water can also be accomplished by making large daily water changes. This method, while efficacious, may stress fish excessively unless attention is paid to makeup water temperature and pH. Alternatively, fish can be removed from a system, and the parasites will eventually die for lack of a host. Elevating the temperature several degrees Celsius over normal temperatures accelerates this process. To ensure that all theronts are eliminated in a system, at least one complete water change should be made, along with removing debris from the gravel before returning fish to the system after leaving a tank or system fallow.

Treatment

Currently available medications do not penetrate the encysted trophonts (Tomonts). All treatment is directed toward preventing reinfection of fish by killing free-swimming theronts. Formaldehyde at 25 ppm (1 ml/10 gallons) is effective if administered three times on alternate days. Water changes of up to 75% should be done 4–8 h after treatments. In addition to chemotherapy, management adjustments serve to control infestations. Elevating water temperatures several degrees Celsius over normal temperatures for 5–7 days will limit the infection by adversely affecting the heat-sensitive theronts as well as enhancing the immune response of the host.

Probable questions:

1. Name the causative agent of whirling disease. Why this disease is named so?
2. Describe the life cycle of *Myxobolus cerebralis* with diagram
3. Describe triactinomyxon spores with diagram.
4. Describe the myxosporean stage of *Myxobolus cerebralis.*
5. Discuss the developmental stages of *Ichthyophthirius multifiliis*.
6. Which life cycle stage of *Ichthyophthirius multifiliis* is highly susceptible to chemicals?
7. Briefly describe the life cycle of *Ichthyophthirius multifiliis*\ with diagram.
8. Write short note on white spot disease.

Suggested readings:

1. http://www.columbia.edu/itc/cerc/danoff-burg/invasion_bio/inv_spp_summ/myxobolus_cerebralis.html
2. https://parasite.org.au/para-site/text/ichthyophthirius-text.html
3. Roberts, L.S and Janovy, J. (2009). Smith & Robert's Foundation of Parasitology. 8th. Edn.McGraw Hill
4. Ahmed, N., Dawson, M., Smith, C.and Wood, Ed. (2007). Biology of Fish Disease. Taylor and Francis Group

Unit III

Some common helminthes of freshwater fishes and their life cycle patterns: A) *Proteocephalus* sp. B) *Camallanus* sp.

Objective: In this unit you will know about some common helminths of freshwater fishes and their life cycle patterns like *Proteocephalus* sp. and *Camallanus* sp.

• *Proteocephalus* sp.

Introduction

Proteocephalid cestodes parasitize fish predominantly but occur also in amphibians and reptiles; their intermediate hosts are primarily planktonic crustaceans. They have distinct segmentation, variable scolex with four simple suckers, an apical sucker or occasionally an armed rostellum. Tapeworms of the genus *Proteocephalus* Weinland, 1858 are parasites of fishes, amphibians and reptiles. The systematics of this genus has not been sufficiently clarified and there are difficulties in the identification of individual taxa.

It has been found that Palearctic species of *Proteocephalus* parasitic in fishes are highly uniform in their overall morphology. On the contrary, several taxa are polymorphic. This results in a shortage of morphological or biometrical features potentially suitable for species identification.

To date, the developmental cycles of only a few *Proteocephalus* tapeworms have been studied and, compared with other cestode groups such as the Pseudophyllidea and Cyclophyllidea, the biology of proteocephalidean tapeworms is much less well known. Life cycles of *Proteocephalus* species occurring in fishes in the Palearctic Region are reviewed with emphasis on the morphology of larval stages and development of parasites within the intermediate and definitive hosts.

Systematic position

Kingdom: Animalia
Phylum: Platyhelminthes
Class: Cestoda
Genus: *Proteocephalus*

Life cycle

The basic sequence of *Proteocephalus* development consists of an *adult*, which producesan *egg*, containing an oncosphere, i.e. a six-hooked (hexacanth) *larva*, which migrates to a parenteral site (body cavity) of the intermediate host, where it metamorphoses and grows as a *metacestode*, and a sexually reproducing adult.

Egg

The egg results from oogenesis, fertilization of the oocyte and subsequent embryogenesis. This process, including the formation of sperm, i.e. spermatogenesis, will be briefly discussed, with emphasis on the morphology of formed eggs.

Spermatogenesis

The male reproductive plan of *Proteocephalus* species is typical of parasitic platyhelminths. Despite the fact that the first study on the ultrastructure of cestode sperm flagellum was that by Gresson (1962) in *P. pollanicola* (syn. of *P. longicollis* –Scholz *et al.*, 1998b), there is limited information about spermatogenesis and sperm ultra-structure of *Proteocephalus* species. It has been found that *P. longicollis* has a spermatozoon and spermiogenesis with the following chief features:

(i) A long thread-like body;

(ii) An elongated nucleus;

(iii) Cortical microtubules underlying the plasma membrane;

(iv) The absence of mitochondria; and

(v) The absence of a typical acrosome.

Because of the presence of two axonemes, *Proteocephalus* seems to belong to the 'two-axoneme' type of cestodes, found typically in the Pseudophyllidea and Tetraphyllidea. This type is considered primitive (plesiomorphic) because it is present in free-living platyhelminths

Oogenesis and insemination

There are few studies on oogenesis and insemination in cestodes, which is valid also for proteocephalideans. Although no data exist, it can be assumed that the chemical composition and ultra-structure of oocytes of *Proteocephalus* species resemble those of other cestode groups.

Egg formation

Most studies on cestode egg formation have dealt with pseudophyllidean and cyclophyllidean tape worms and practically nothing is known about the process of formation of egg envelopes of species of *Proteocephalus*. Four main types of egg-forming systems are recognized in cestodes and proteocephalidean tapeworms are considered to belong to the 'pseudophyllidean- type'. Cestodes with life cycles associated with water are placed in this group.

Many of them, in particular pseudophyllideans, have a thick, sclerotin capsule, produced by cestodes with well-developed vitellaria. In this feature, proteocephalideans distinctly differ from these groups in possessing a thin walled, transparent outer envelope. Eggs of proteocephalideans are named as 'egg-like oncospheres' to distinguish them from coracidia of pseudophyllideans. In pregravid proglottides of *Proteocephalus* species, all eggs contain an unformed oncosphere, i.e. there are no embryonic hooks. The hooks appear simultaneously in most eggs in more developed proglottides, which are named gravid. The number of pregravid proglottidesis highly variable in *Proteocephalus* species, ranging from a few to numerous.

Egg morphology

Eggs of *Proteocephalus* species are similar in their overall appearance and are composed of an oncosphere covered by membranes (fig. 1).

The oncosphere (hexacanth), already formed within the uterus of gravid worms (Fig 1A), contains three pairs of embryonic hooks with a straight, long and fine base, a short, slightly curvedblade and a short, anteriorly directed guard. Hooks of the median pair are longer than those of lateral pairs. Two dark areas considered to be penetration glands, are situated on the opposite side of the oncosphere to the embryonic hooks. These are found in the morphology of the embryonic hooks of *P. exiguous*, *P. percae* and *P. thymalli* from Russia. They found significant differences in the size of the hooks of

individual taxa studied. However, differences were also found between different populations of the same species.

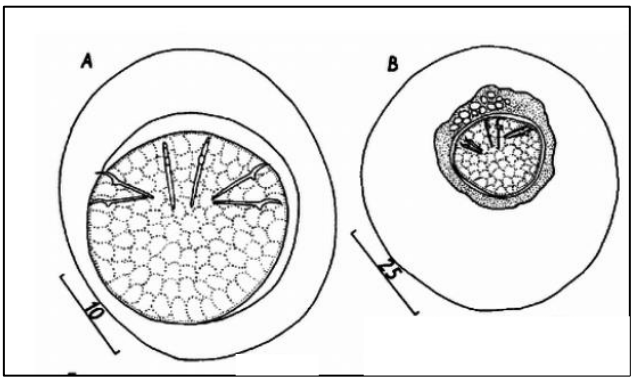

Fig 1: A, oncosphere with internal membranes; B, ripe egg

The differing lengths of embryonic hooks were also used for distinguishing procercoids of *P. macrocephalus* and *Proteocephalus* sp. (most probably *P. longicollis*) in naturally infected copepods. It is recommended that further investigations are performed in order to confirm the suitability of embryonic hooks for species differentiation, as in oncospheres of pseudophyllidean tapeworms of the genus *Diphyllobothrium* (Hilliard, 1960).*Proteocephalus* oncospheres probably have no flamecells, but these are present in oncospheres of some tapeworms, e.g. in *Diphyllobothrium*. A formed oncosphere of *Proteocephalus* tapeworms is enclosed in layers. Three basic embryonic envelopes can be distinguished:

(i) Capsule (¼ egg shell);

(ii)Outer envelope; and

(iii) Inner envelope, which is asyncytial layer showing much variation and giving rise to the oncospheral membrane.

Egg size

The size of eggs has been used as an important feature in differentiating species of *Proteocephalus.* However, data taken from eggs in permanent preparations are of limited value because the eggs are generally deformed due to staining and dehydration; the capsule is collapsed so that most measurements provided in this study seems to relate to the outer envelope. It has been suggested that only measurements of eggs expelled from the uterus into the water should be measured. However, only ripe eggs, i.e. those containing fully formed and motile oncospheres with embryonic hooks, should be considered. It should also be pointed out that the capsule of most *Proteocephalus* tapeworms inflates in water which makes it difficult to compare with measurements of eggs present at different times in water. Therefore, it seems reasonable to provide measurements of a more stable structure, i.e. those of the outer envelope covering the rigid, granular layer, and of the oncospheres.

Egg release

Ripe eggs, i.e. those containing oncospheres with embryonic hooks (hexacanth), are released through the uterine pores. In *Proteocephalus* species, the uterine pores are few (about 2–4), small, oval to spherical openings along the median line on the ventral side of gravid proglottides. To date, no data on the formation of uterine pores have been provided. Eggs are spontaneously released after the tapeworms are placed in water and it seems that this release corresponds with the strategy of egg release under natural conditions. It has been observed that eggs of some species, e.g. *P. osculatus* and *P. torulosus*, are released directly within the lumen of fishes. However, it is possible that this release

is linked with the death of the host and it does not occur in nature. Outer membrane of egg is thin and apparently unable to prevent digestion by host enzymes in the anterior part of the intestine, the site of location of adult tapeworms. The eggs, protected against unfavourable conditions of the intestinal lumen within the uterus of the worm, can reach the external environment with more safety by expulsion of the whole egg-containing parasite or by expulsion of the detached part of the strobili. Indeed, the body of gravid *Proteocephalus* is often fragmented within the intestinal lumen.

Another way of expelling eggs was observed in *P. filicollis* tapeworms partly protruded from the anus and expelling eggs through the uterine pores. Stimuli of egg release have not been studied in detail but it is likely that the most important is direct contact of gravid worms with water. Eggs are released from proglottides quite rapidly and a large amount of eggs is expelled in a few minutes. However, no information exists on the fecundity of *Proteocephalus* tapeworms.

After the egg is laid in water, the external membrane quickly increases in size, becoming two or three times larger. It is presumed that this process is due to osmotic intake of water. It is also assumed that the swelling of the capsule helps in the floating of the egg because freshly laid eggs remained on the bottom whereas the eggs with inflated capsules may float in the water. Since intermediate hosts are mostly pelagic copepods, this is an adaption to facilitate transmission by selective ingestion. Eggs of *P. torulosus* are relatively large and they differ from those of congeners in possessing a somewhat thicker granular layer and a less inflated capsule. This is probably an adaptive character because *P. torulosus* is a riverine species, occurring even in rivers with strong current. In these localities, eggs which sink to the bottom are likely to survive for successful transmission to potential intermediate hosts, such as benthic copepods.

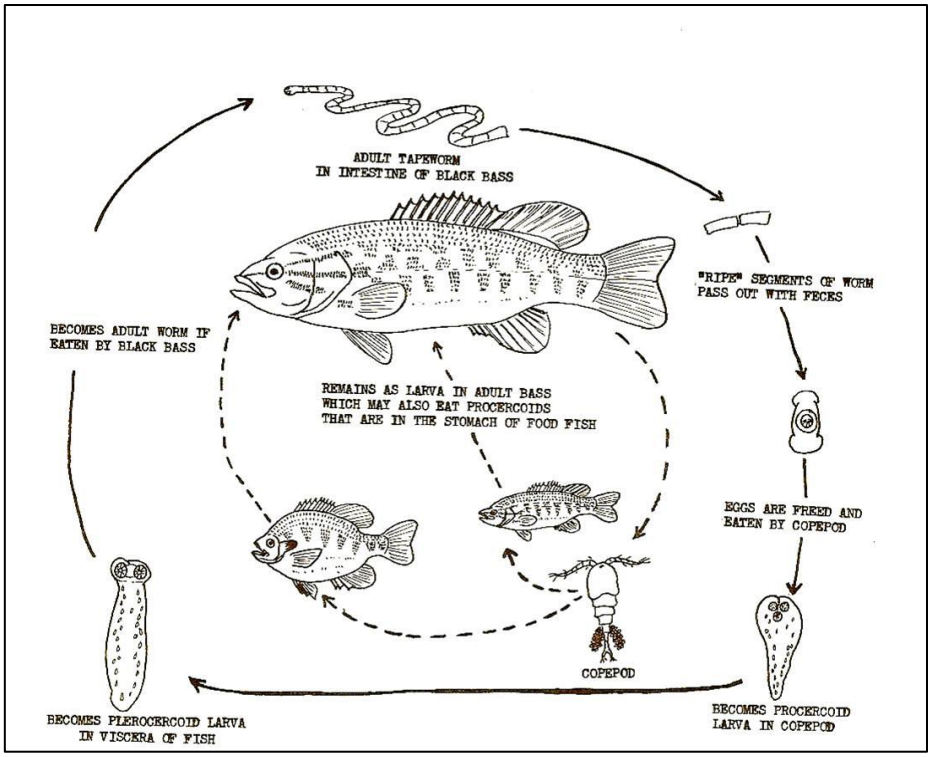

Fig 2: Life cycle of *Proteocephalus*sp.

Survival and infectivity

It has been observed that not all eggs spontaneously laid in water are ripe. Some eggs are apparently smaller than others and contain either unformed oncospheres, i.e. without embryonic hooks, or undifferentiated, granular tissue. The proportion of ripe eggs of *P. exiguus* was found to change

during the year with the highest proportion in summer–autumn but only very few during the winter and spring. Infectivity, i.e. the ability of oncospheres to infect intermediate hosts, was tested by experimental infections of copepods with eggs preserved in water of different temperatures. It has been demonstrated that eggs maintain their infectivity for a relatively long time and that infectivity depends mainly on the temperature. Only freshly released *P. torulosus* eggs are infective to copepods and that they lose their infectivity very quickly seem to be incorrect because they contradict successful experimental infections with *P. torulosus* eggs several days old.

Intermediate host

Range of intermediate hosts

Planktonic crustaceans of the order Copepoda (families Diaptomidae and Cyclopidae) serve as intermediate hosts (Fig 2) for *Proteocephalus* tapeworms in the Palearctic Region (table 1). An exception to this rule is the calanoid *Epischura baicalensis* (Temoridae), which is an intermediate host of *Proteocephalus* species in Baikal Lake, Russia. The finding of *Proteocephalus* larvae in cladocerans (*Bosmina coregoni*, *Bythotrephescederstroemi*, *Daphnia* spp.) should be considered as accidental or even doubtful. The eggs ingested by cladocerans survived within the intestine for a short time (maximum 48 h) but oncospheres were unable to penetrate through the intestinal wall and quickly died. The suitability of individual copepod species as intermediate hosts of *Proteocephalus* species differs and is dependent upon the species and developmental stages of copepods as well as particular ecological conditions such as locality and season. Some copepods tend to be more susceptible to *Proteocephalus* infection than others but data which would explain these differences are not available.

The different developmental stages of copepods have also distinct susceptibilities to infection with *Proteocephalus* oncospheres. Nauplii of *C. strenuus* to be more heavily infected with *P. exiguus* (= *P. longicollis*) procercoids than other developmental stages (copepodites and adult copepods)(prevalence 64%). This difference is due to presence of a thinner gut wall in nauplii compared to that in adult copepods.

Table 1. Survey of natural (N) and experimental (E) intermediate hosts of *Proteocephalus* species. With the exception of *Epischura baicalensis* (Calanoida), all intermediate hosts belong to the families Diaptomidae and Cyclopidae (Copepoda).

Species	Intermediate host
P. ambiguus	*Eudiaptomus gracilis (N), Cyclops strenuus*
P. cernuae	*Cyclops strenuus (E)*
P. longicollis	*E. gracilis (N,E), E. graciloides (N), E. zachariasi (N), Cyclops furcifer (E), C. kolensis (N,E), C. lacustris (N),*
P. filicollis	*E. gracilis (N), C. strenuus (E), Eucyclops serrulatus (E), Mesocyclops oithonoides (N,E)*
P. osculatus	*C. strenuus*
P. torulosus	*Diaptomus castor (E), E. gracilis (N), Heterocope appendiculata (N), C. strenuus (N,E), Cyclops sp., Eucyclops*
Proteocephalus sp.	*Cyclops abyssorum (N,E), C. strenuus (?N,E), C. vicinus (E), Epischura baicalensis*

Infection of the intermediate host

Copepods are attracted by floating eggs and ingest them quickly but other authors suggest that copepods reject oncospheres and the consumption of eggs is occasional and accidental. The results of experimental infections, indicating high prevalences in infected copepods, support the assumption of attraction rather than accidental ingestion of proteocephalidean eggs. It can be assumed that eggs of *Proteocephalus* species, because of their size, are accessible to most planktonic copepods because they are found in young developmental stages such as the nauplii and copepodites.

After ingestion, oncospheres are liberated from surrounding membranes in the gut of copepod, and presumably the release of the larvae in the intestine is stimulated by the environment of the intestinal lumen. The process of liberation is rather rapid and oncospheres appear to be free of the egg membranes as early as 5 min after contact with the copepods. This liberation of the oncospheres has also been observed in eggs in water and it can be stimulated by applying slight pressure. However, those oncospheres which are liberated directly into water do not survive.

The success of oncosphere establishment within copepods and the proportion of intermediate hosts becoming infected are influenced by many factors, including physiological compatibility, ecological conditions and the geographic origin of the host and parasite, the time of contact of copepods with eggs, the density of copepods, and water temperature.

Following ingestion, oncospheres are liberated from egg membranes and, if in a suitable host, actively penetrate the gut of the copepod into the body cavity. Penetration of oncospheres though the gut wall is assisted by the mechanical action of embryonic hooks. Freeman (1973) assumes that these hooks may be used more for attachment to the gut wall, and that secretions facilitate a more passive less disruptive penetration than that resulting from ‘clawing’. The secretion of penetration glands, with presumably histolytic secretions, may play a crucial role in the process of penetration. The time of penetration of the gut wall is short, lasting 5–30 min.

Within the body cavity, oncosphere develops into a metacestode. A number of terms have been used to describe *Proteocephalus* metacestodes in the intermediate host. These include: plerocercoid, plerocercoid I, cercoscolex or procercoid. The term ‘plerocercoid’ has usually been restricted to pseudophyllidean metacestodes from the second intermediate host.

In the genus *Proteocephalus*, all metacestodes from intermediate hosts as well as juvenile worms from the definitive host were regarded as plerocercoids. The metacestode of *P. filicollis* from a copepod intermediate host is named ‘caudate culcitacetabuloplerocercoidI’ and that of *P. ambloplitis* ‘a caudate invaginated glandacetabuloplerocercoid I’. However, this terminology is very complicated and it has not received general acceptance among helminthologists. Jarecka (1975) proposed a simple terminology for the larval stages of tapeworms but she only dealt with three cestode orders, namely Pseudophyllidea (including the caryophyllideans), Proteocephalidea and Cyclophyllidea. She divided cestodes into ‘oviparous’, i.e. those possessinga coracidium (Pseudophyllidea) and ‘viviparous’. It appears to be appropriate to regard an acystic metacestodes from the first intermediate host as a ‘procercoid’ rather than a ‘plerocercoid’ or ‘cercoscolex’.

Morphogenesis of procercoids

Procercoid morphogenesis within the intermediate host is similar in all species hitherto studied The development of *P. cernuae* within Cyclops strenuus at 20–22 °C is briefly described, with remarks on other species. Within the body cavity, i.e. in the parenteral site, the oncosphere quickly grows due to cellular proliferation and it metamorphoses into the developing metacestode. However, no precise data exist on the process of metamorphosis of the oncosphere into the metacestode, i.e. when

resorption of oncospheral structure, if it occurs, is completed. The metacestode becomes elongate 4–6 days post infection with one end more actively moving. On the opposite side, a small protuberance, ultimately becoming detached from the body and representing the primordium of a cercomer, appears 5–8 DPI. The cercomer is formed 7–9 DPI as a small spherical appendix connected to the body by a narrow stem. The cercomer detaches from the body after 1–3 days or persists for three weeks. A detached cercomer can remain viable within the body cavity of a copepod for as long as 60 days. A cercomer has not been observed in *P. filicollis* and *P. torulosus* metacestodes and in North American species *P. ambloplitis* and *P. pinguis*.

The presence/absence of the cercomer has important phylogenetic consequences according to Freeman (1973), because he proposed that two main lineages of proteocephalideans and cyclophyllideans evolved from primitive proteocephalideans. The cercomer of *P. exiguus* and *P. neglectus* metacestodes was described as containing embryonic hooks. However, embryonic hooks are normally located within the body, most often near its lateral margins in procercoids of the same species and those of other Proteocephalus taxa. The location of hooks within the cercomer is apparently exceptional. In this extra cercomeral location of embryonic hooks, the procercoids of Proteocephalus species differ from those of most pseudophyllidean tapeworms, in that the cercomer of which contains embryonic hooks. However, the extra cercomeral position of embryonic hooks in Proteocephalus metacestodes is not unique among cestodes. Notwithstanding the final position of embryonic hooks in a metacestode, it is suggested that they have no further role in the subsequent development of the cestode. In some cestodes, a distinct cavity, the 'primitive lacuna', develops whereas other metacestodes grow as a compact mass of cells. The presence of a cavity or 'lacuna primitiva', it seems that in Proteocephalus a cavity normally does not develop and metacestodes are acystic and gymnosomic. Within 7–8 days, the primordium of an apical sucker appears in *P. cernuae* procercoid slightly before or simultaneously with the primordium of the lateral suckers. Development of the scolex may vary considerably; the appearance of a single structure at the extreme tip is often the first sign of scolex differentiation during the exogenous development of acystic metacestodes. Suckers develop quickly in Proteocephalus procercoids, being well formed 9–10 DPI in *P. cernuae*. Calcareous corpuscles are typical features of metacestodes and may persist in juvenile tapeworms in the definitive host, their function is still unknown. It is assumed that they play an important role in the metabolism of early developing intestinal worms and they buffer anaerobically produced acids and gastric hydrochloric acid. Regarding the longevity of procercoids within the intermediate host, some larvae are able to survive until the death of copepods, at least 2–2.5 months at 17–20°C.

Morphology of procercoids

Fully formed (infective) procercoids are elongate and highly mobile. The shape and size of procercoids vary considerably due to their high motility and there is also much individual variation in size. The size of procercoids is influenced by the species and stage of intermediate host and the intensity of infection. High individual and intraspecific variability exists in the size of fully developed procercoids of Proteocephalus species, with the length ranging from 140 to 730mm and maximum width between 50 and 150mm.

The procercoid possesses a well-developed anterior part (scolex) with four muscular suckers. Although the scolex does not reach the ultimate size of that of the adult worm, its morphology generally does not differ from that of tapeworms from the definitive host. The procercoid of *P. osculatus* has a well-developed, functional apical sucker with a deep cavity. In other species (*P. cernuae, P. filicollis, P. longicollis, P. macrocephalus*) the proceroid possesses just a vestigial, but distinct apical sucker, with its morphology similar to that in the adult. The procercoids of *P. torulosus*,

similar to adult worms of this species, possess no apical sucker but numerous glandular cells concentrated in the apical part of the scolex. The procercoid body is covered with well-developed microtriches. The excretory system of *Proteocephalus* metacestodes consists of flame cells, secondary canals and two pairs of main collecting ducts united posteriorly and opening by ventral ducts into an elongate, thick-walled excretory bladder. In the anterior part of the body, the main ducts divide into secondary canals forming a dense network mainly around the lateral suckers.

The procercoid body contains numerous calcareous corpuscles of variable shape, measuring 4–14mm in length; the corpuscles persist in the body of juvenile tapeworms within the definitive hosts but their number rapidly decreases. A uniform morphology of *Proteocephalus* procercoids makes it difficult to identify them specifically. No primary cavity develops within the body of *Proteocephalus* procercoids during their formation. Therefore, this type of development is primitive according to the classification of Freeman (1973) and corresponds to that typical of other 'lower' cestode orders, such as the caryophyllideans and pseudophyllideans.

Localization of larvae

Larvae are freely moving within the body cavity of copepods but they are located most frequently in the first segments of the cephalothorax. At the beginning of development, larvae can also be located in the antennulae, followed by exclusive development within the cephalothoracic or abdominal segments, apparently due to space limitation in the antennulae. Migration of larvae from the antenulae to the body cavity after 3 days of development but Priemer (1980) found *P. neglectus* metacestodes in the antennulae of *C. strenuus* as late as 13 days after infection at 98°C. Only insignificant changes in the site preference of *P. neglectus* metacestodes within the body cavity of copepods were observed during their development.

Occurrence in intermediate hosts

Values of prevalence reach up to 100% in experimentally infected copepods but in natural populations of conspecific copepods the prevalence values are considerably lower. Generally, the prevalence of infection of zooplankton with Proteocephalus procercoids (and other metacestodes) is extremely low, ranging between 0.001 and 1%. Although the prevalence values of copepod infection under natural conditions are very low, parasites can accumulate within fish hosts due to an intensive consumption of zooplankton by fishes. The absolute number of Proteocephalus procercoids in naturally infected copepods can reach 853–1193 specimens per m^3 with mean values 3–178 specimens per m3.

The prevalence of infection markedly fluctuates under natural conditions, being dependent on factors such as the species of copepod infected and their developmental stages, seasonality and locality Seasonal patterns in the occurrence of *Proteocephalus* metacestodes in naturally infected copepods depend on the time of egg release, which is controlled mainly by water temperature. It has been observed that intermediate hosts are infected almost exclusively in the summer or early autumn.

It is also assumed that Proteocephalus procercoids survive diapause in the copepods. The role of individual copepod species changes during the year with a gradual substitution of copepod species more susceptible to infection by less susceptible ones. This appears to be related to seasonal changes in the occurrence of potential intermediate hosts and their availability. More susceptible copepods may be absent when cestode eggs are released into water and thus less susceptible species of copepods may play an important role as intermediate hosts.

Definitive host

Range of definitive hosts

Host specificity of most *Proteocephalus* species from fishes has been considered to be quite narrow but there are marked differences in the range of fish hosts infected by individual species. Some species are specific to one host genus or one species of definitive host, e.g. *P. ambiguus* to the nine-spined stickleback (*Pungitius pungitius*), *P. filicollis* to the three-spined stickleback (*Gasterosteus aculeatus*), *P. macrocephalus*nto eels (*Anguilla* spp.), *P. osculatus* to wels (*Silurus*n*glanis*), and *P. thymalli* to graylings (*Thymallus* spp.).nOther taxa, however, occur in a variety of fish species of one or more families: *P. gobiorum* in gobiids (Gobiidae), *P. longicollis* in salmonid fishes (Coregonidae, Salmonidae),*P. percae* in percids (Percidae), *P. tetrastomus* in smelt (Osmeridae), and *P. torulosus* in cypriniform fishes (Cyprinidae and Cobitidae).

Infection of the definitive host

Definitive hosts become infected after ingestion of copepods harbouring procercoids. Larvae continue to develop within the digestive tract of the fish host, with subsequent formation of attachment organs, nervous and excretory systems and musculature. The growth of these organs occurs within the definitive host but considerable changes in scolex morphology or structure of the osmoregulatory system do not occur. The morphology of the scolex of procercoids closely resembles that in the adult worms and its morphology plays a crucial role in the process of establishment of tapeworms within the definitive host.

Scolex of living Proteocephalus larvae becomes invaginated immediately after ingestion by the definitive host and this is related to the parasite being protected against unfavourable conditions such as the high acidity within the stomach of the fish definitive host. Procercoids become more active and the scolex evaginates in alkaline conditions, so the role of chemical stimuli in the process of evagination requires further investigation.

The invagination of the scolex has also been observed in procercoids in experimentally infected copepods and in larvae artificially isolated from the body cavity of intermediate hosts and maintained in water or saline. Metacestodes are readily affected by changes in the osmotic pressure of the medium but they remain mobile within the copepod haemocoel.

Only a very small proportion of juvenile tapeworms are able to establish within the gut of the definitive host. Some time (‘physiological maturation’) after complete formation of internal organs is necessary for procercoids to become fully infective. Intraspecific competition between young worms within the pyloric caeca or in the intestine of infected fish is likely to occur but the very low establishment rate requires additional studies.

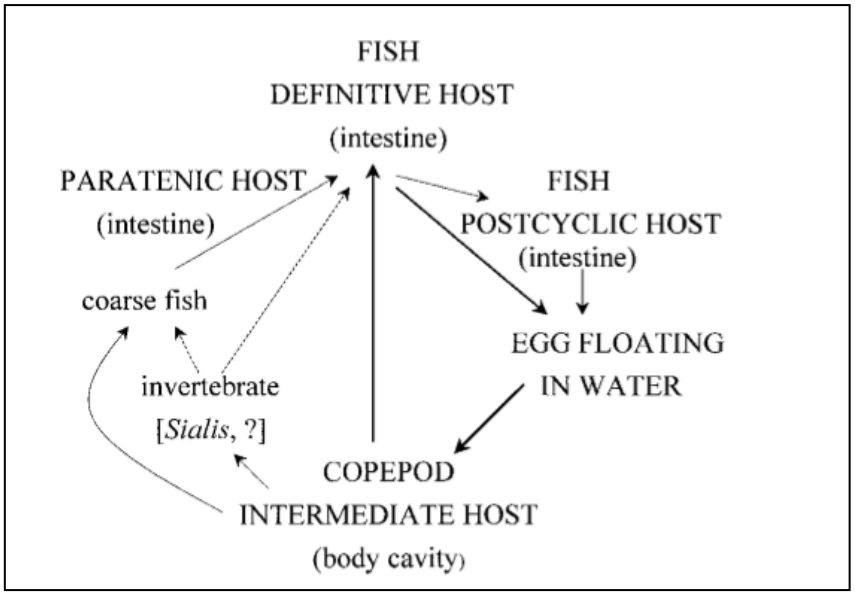

Fig: Flow diagram of the life cycles of Proteocephalus tapeworms. Dotted line indicates possible routes of transmission

Maturation dynamics

As in the case of the development of procercoids in copepods, the growth and maturation of tapeworms in the fish definitive host are controlled mainly by water temperature. The influence of host hormones, as observed in some pseudophyllideans, such as Triaenophorus spp. may also play some role. Although it is possible that one cycle may be completed in 1.5–2 months at water temperatures of 15–20 ° C. *Proteocephalus* have a one year life span (i.e. the life cycle in total, including all developmental stages) with a pronounced seasonality in their maturation. The recruitment of new cestode generations takes place mainly in the summer or autumn. The tapeworms overwinter in fish and they start to grow rapidly and mature after the water temperature increases in the spring. Eggs are laid in late spring and summer. Such seasonal patterns in occurrence and maturation have been observed in several taxa of Proteocephalus, e.g. *P. cernuae, P. exiguous, P. filicollis, P. osculates*. This general pattern is modified in each species, being dependent on its geographical position and particular ecological conditions, as the same species of cestode may show different patterns of maturation in distinct latitudes. Localization within the definitive host, as a rule, adult Proteocephalus tapeworms is located in the anterior part of the intestine. In fishes possessing pyloric appendages, adult tapeworms are attached by their scoleces to the epithelium of these appendages with the strobilae lying within the intestinal lumen.

Conclusions

The most detailed information exists on the biology of *P. longicollis* but also in this species many aspects of its life history remain to be studied. There are still gaps in our knowledge of biology of Proteocephalus tapeworms parasitizing fishes in the Palearctic Region and further investigations into the life cycles are needed. It is difficult to list unsolved problems in the biology of Proteocephalus tapeworms, which should be addressed in future research, but the following deserve attention, namely the process of egg formation, including fertilization; ultra-structure of eggs and oncosphere, with special attention being paid to the penetration glands; comparative morphology of embryonic hooks; morphogenesis and ultra-structure of the procercoids, in particular those of *P. gobiorum* and *P. tetrastomus*; factors influencing the establishment of juvenile tapeworms and their morphogenesis

within the definitive host; factors resulting in the host specificity of some taxa in the definitive host. Knowledge of tapeworm biology is still fairly limited despite the considerable progress being made in the past two to three decades.

• *Camallanus* sp.

Introduction

The nematode *Camallanus anabantis* Pearse, 1933 is the common intestinal parasite of *Anabas testudineus* (Bloch) (type host) and also reported to be harboured by many other fishes such as *Clarias batrachus* (Linnaeus), *Channa gachua* (Hamilton), *C. punctatus* (Bloch), *Puntius filamentosus* (Valenciennes), *Betta unimaculata* (Popta), *Mastacembelus armatus* (Lacepede) and *Trichogaster trichopterus* (Pallas). *Camallanus anabantis,* this species is common in various fishes in India, including the type host *Anabas testudineus* from freshwater swamps (Soota, 1984; De, 1993). Invasion of fish occurs in spring and summer and they grow in the fish during the monsoon and autumn. The proportion of males in the worm population increases in the early autumnal period and then decreases rapidly after they have fertilized the females. Larvae are released from females in late winter and early spring.

Systematic position

Phylum: Nematoda
Class: Secernentea
Subclass: Spiruria
Order: Camallanida
Family: Camallanidae
Genus: *Camallanus*

Life cycle

Intermediate hosts

Larvae show considerable motility and become ingested by copepod, *Mesocyclops leuckarti*s, and migrate from its digestive tract to the haemocoel of the cephalothorax, where they develop further. The first-stage larvae increase in size, both the cuticle and wall of the oesophagus become thickened and consequently, the spacious oesophageal cavity narrows. The oesophageal gland cells with distinct nuclei appear at the posterior end of the oesophagus. The first moult occurred on day 4, at 21-26°C. The second moult, to attain the third infective-stage, occurred on day 11, at 21-26°C. The fully developed third-stage larvae were, however, first recovered from the copepod hosts on day 14. They remain in the haemocoel of the cephalothorax of the copepod intermediate host as coiled third-stage larvae and their further development ceases.

Definitive hosts

The nematodes were found located in the caecum (third- and fourth-stage only) and anterior and middle parts of the host's intestine. Morphological data of the nematodes recovered from the fishes

revealed that the third moult occurred on day 15. The fourth i.e. final moult occurred at different times for the “male” and “female” larvae, on day 68 in “male” larva but on day 86 in “female” larva. A female with few larvae in the uteri was recovered on day 187.

Morphology and larval development

First-stage larvae

Body of the first-stage larvae (Fig A1) is translucent, 231-300 long and 13-15 in maximum width Body cuticle transversely striated. Head rounded, with a small dorsal cuticular tooth; oral papillae indistinct. Short buccal tube. Oesophagus thin-walled and with spacious lumen occupies 15-19% of body length. Nerve-ring encircles oesophagus at its posterior third. Excretory pore anterior to nerve

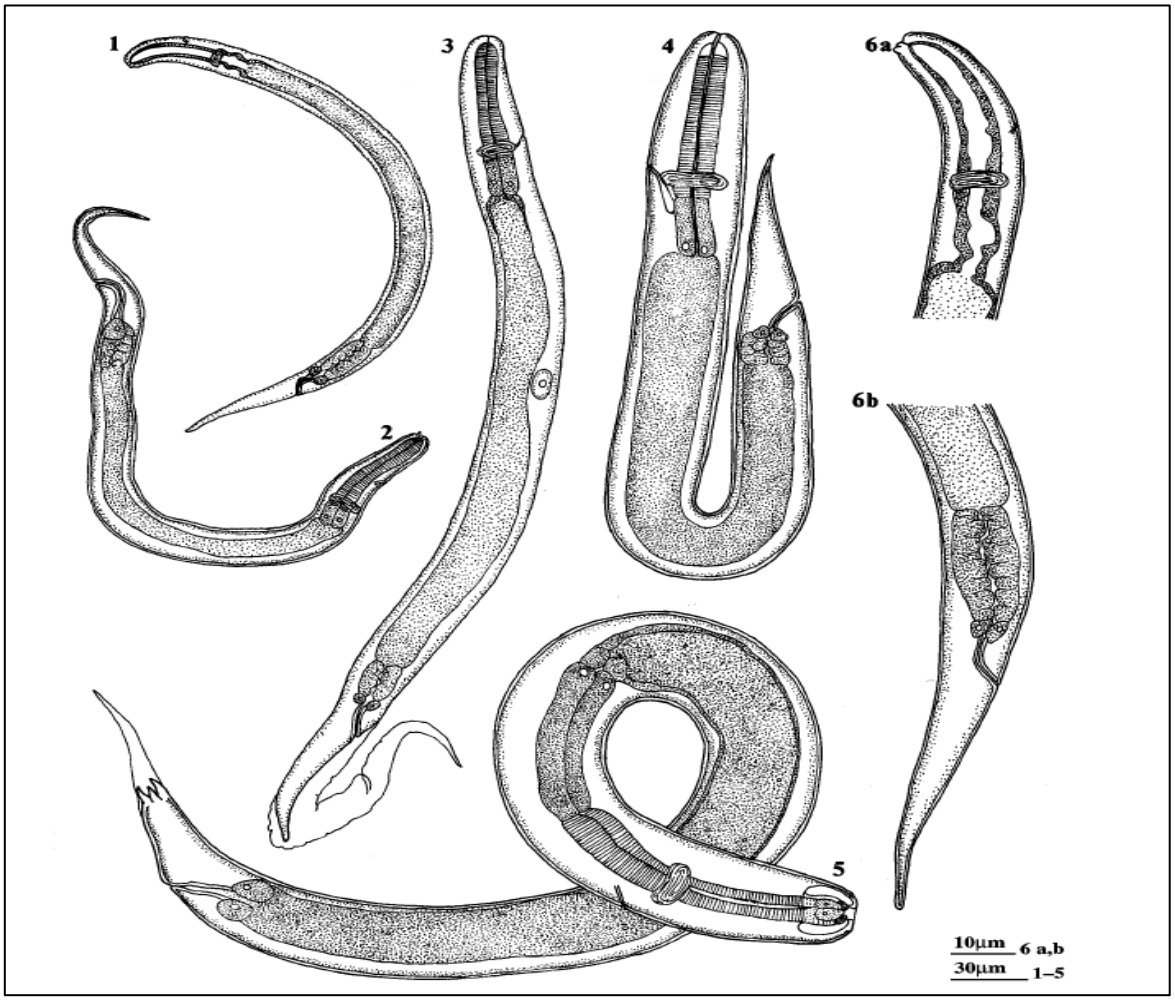

Fig A: Development of larva of *Camallanus anabantis*
(1) Free first stage larva
(2-6) Development of larva in copepod
(2) First stage larva on day 2
(3) Larva during first moult on day 4
(4) Second stage larva on day 7
(5) Late second stage larva on day 10
(6) Free first stage larva, enlarged view (a- anterior end of boby, b- tail end)

ring. Intestine wide, light-coloured and with fine granules; posteriorly with large cuboidal cells. Tail conical, elongate with pointed tip, 13-19 % of body length. First-stage larvae (Fig A2) after

penetration into the haemocoel of the copepod, the dimensions of first-stage larvae changed only a little but the oesophagus differentiated into a thick-walled anterior moiety and a posterior glandular moiety with distinct cell nuclei.

Second-stage larvae

The first larval moult (Fig A3) occurred in the haemocoel of copepod, *Mesocyclops leuckarti* on day 4. At this stage larva measures 435 long and bears no dorsal cephalic tooth. Oesophagus indistinctly divided into anterior muscular (58 long) and posterior glandular (21 long) parts. Intestine straight and wide, a short posterior part with large cuboidal cells and narrow lumen. Small oval ventral genital primordium near midintestine. Tail relatively short, 14% of body length.

Second-stage larvae (Fig A4, A5, B9) were first recovered on day 7. Body more stout, tail relatively short. Cuticle transversely striated. Cephalic end rounded and without dorsal cuticular tooth; buccal tube short. Oesophagus with anterior long muscular and posterior short glandular moieties. Straight, wide intestine with short posterior zone bearing large cuboidal cells.

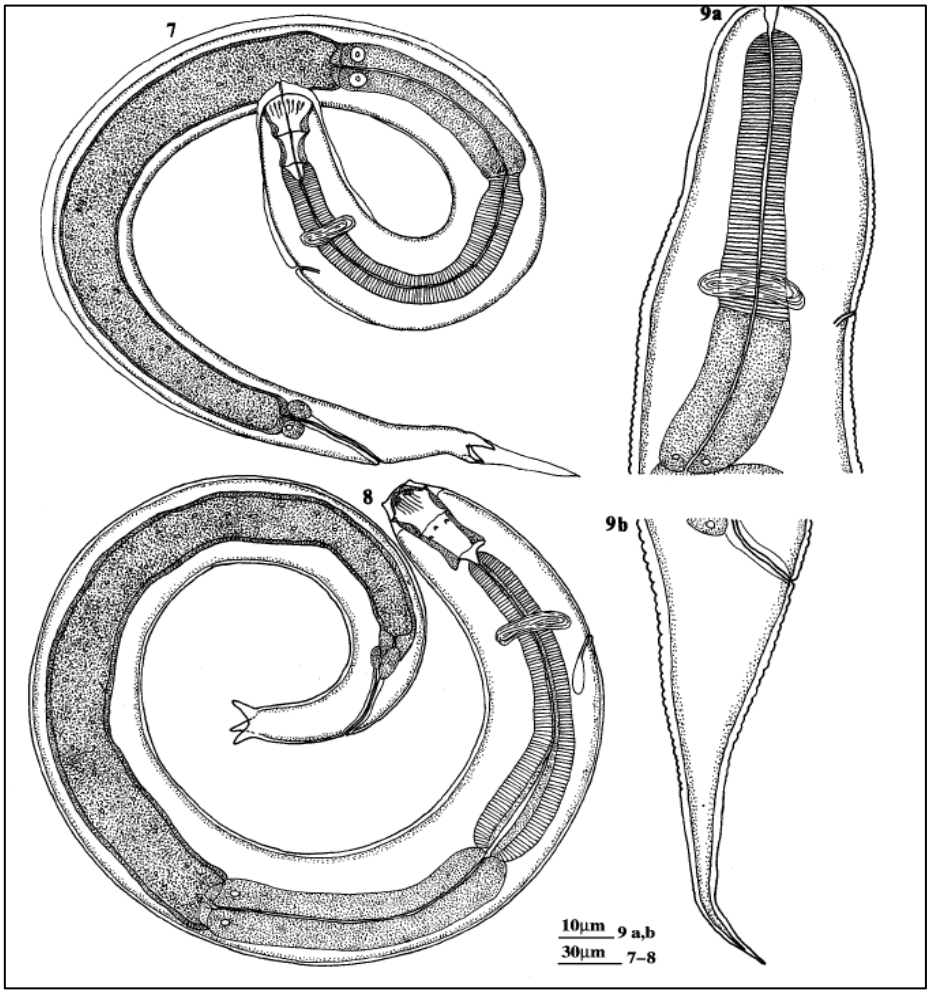

Fig B: Development of larva of Camallanus anabantis in copepod
(7) Larva undergoing second moult on day 11
(8) Third stage larva on day 15
(9) Second stage larva on day 6 (a - anterior end, b - posterior end)

The larva recovered on day 10 was found preparing for its second moult, best visible at tail end. The typical third-stage tail visible below larval cuticle. Anterior end of oesophagus with a hyaline bell-

shaped structure, two small bead-like structures and two large cells with distinct nucleus also visible. Oesophagus with anterior muscular moiety longer than posterior glandular one. Nerve-ring near middle of muscular oesophagus. Excretory pore slightly posterior to nerve ring. Intestine straight, wide and with coarse granules.

Third-stage larvae from copepods

Larvae (Fig B7) undergoing the second moult were recovered from the copepod, *Mesocyclops leuckarti,* haemocoel on day 11. During moulting, old cuticle becomes loosened at both ends, particularly on tail. Larval body light-coloured and covered with thin cuticle. Oral opening dorsoventrally elongate. Buccal capsule, typical camallanid type, already formed and contains two parts; anterior part bivalved, each valve reinforced with 7 sclerotised longitudinal ridges on inner surface and posterior part simple leading posteriorly to an oesophageal cup. Sclerotised inner lining of anterior part of oesophagus seen to pass out through buccal capsule. Oesophagus with longer anterior muscular and short posterior glandular parts, Nerve-ring at about ¼ of oesophagus length. Excretory pore slightly posterior to nerve-ring. Glandular oesophagus opens into intestine through valvular apparatus. Intestine wide, light orange in colour and posteriorly joins short, narrow rectum; three small unicellular rectal glands present at their junction. Tail ends in one dorsal and two subventral mucrones.

Fourth-stage larvae

The single larva undergoing the third (first in the definitive host) moult was recovered from an experimentally infected fish on day 15; its body longer than third-stage larvae from copepods. Cuticle thin and indistinctly striated. Apart from the buccal capsule, typical of the third stage, a new buccal capsule seen. Newly formed buccal capsule weakly sclerotised, wider and not divided into anterior and posterior chambers. Longitudinal ridges on inner surface of new buccal capsule not seen. Tail conical with three mucrones, one large dorsal and two smaller subventral.

Young adult, fifth stage

The fourth moult of "male" larvae first occurred on day 68 at water temperature 24-36°C. Body cuticle is thin. Both old, typical of fourth stage, and new wider and weakly sclerotised buccal capsule with faint beaded ridges in each valve seen. Dorsal and ventral aggregation of large cells found below newly formed buccal capsule. Muscular oesophagus shorter than posterior glandular one. Nerve-ring at about 1/3 of muscular oesophagus. Excretory pore just posterior to nerve-ring. Intestine straight and wide. Tail conical, bears two mucrones. Single genital anlage differentiated into testis and vas deferens. Spicules indistinctly visible but caudal papillae seen clearly.

The first two moults take place in the intermediate copepod host, and the next two, i.e. third and fourth moults, in the definitive host. The morphology of the first- and second-stage larvae of *C. anabantis* is found to be almost identical to that of corresponding larval stages. An interesting feature of the present first- and second-stage larvae is that the intestinal wall at its distal part is made up of large cuboidal cells and therefore, the lumen is narrow there. The larvae at their third stage, however, show some differences, particularly in the structure of the buccal capsule.

Probable questions:

1. Discuss about the egg of *Proteocephalus* sp.
2. Describe the egg morphology of *Proteocephalus* sp.
3. Name a species of *Proteocephalus* found in river water.
4. Name the infective stage of *Proteocephalus* sp.
5. Describe the morphology of procercoids of *Proteocephalus* sp.
6. Describe the development of *Proteocephalus* sp. in definitive host.
7. Describe the life cycle of *Proteocephalus* sp. with diagram.
8. Describe different stages of larval development in *Camallanus* sp.
9. Discuss the life cycle of *Camallanus* sp.

Suggested readings:

1. Scholz, T. (1999). Life cycles of species of *Proteocephalus*, parasites of fishes in the Palearctic Region: a review. *Journal of Helminthology*, **73**: 1–19.
2. Wagner, E. D. (1954). The life history of *Proteocephalus tumidocollus* Wagner, 1953, (Cestoda), in rainbow trout. *Journal of Parasitology,* **40:** 489–498.
3. De N.C (1999). On the development and life cycle of *Camallanus anabantis* (Nematoda: Camallanidae), a parasite of the climbing perch, *Anabas testudineus. Folia parasitological,* 46: 205-215.
4. Ahmed, N., Dawson, M., Smith, C.and Wood, Ed. (2007). Biology of Fish Disease. Taylor and Francis Group

Unit IV

Structure, pathobiology, prophylaxis and diagnosis of causative agents of filariasis; Parasitic insects: *Cimex lectularius and Xenopsylla cheopis*

Objective: In this unit you will know about Structure, pathobiology, prophylaxis and diagnosis of causative agents of filariasis as well as two parasitic insects: *Cimex lectularius and Xenopsylla cheopis*

- **Agents of filariasis**

Introduction

Filariasis is caused by the worms *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. These worms occupy the lymphatic system, including the lymph nodes; in chronic cases, these worms lead to the syndrome of elephantiasis.

- *Wuchereria bancrofti*, which is responsible for 90% of the cases
- *Brugia malayi*, which causes most of the remainder of the cases
- *Brugia timori*, which also causes the disease.

Due to high frequency here we will discuss about *Wuchereria bancrofti*.

Geographic Distribution

Wuchereria bancrofti is largely confined to the tropical and sub-tropical countries of the world. However, it occurs in India, West Indies, Puerto Rico, Southern China, Japan, Pacific Islands, West and Central Africa and South America. In India, the parasite is chiefly distributed along the sea coast and along the banks of big rivers (except Indus); it has also been reported from Rajasthan. Punjab, Delhi and from various vicinities of Uttar Pradesh.

Habit and Habitat

Wuchereria bancrofti is a dreadful endoparasite of man; adults harbouring the lymphatic vessels and lymph nodes. Its life history is digenetic, as it involves a secondary host, the bloodsucking insects, i.e., the female mosquitoes of the genus *Culex*, *Aedes* or *Anopheles*; the secondary host for *W. bancrofti* in India and China is *Culex pipiens*, in Pacific Islands (except Fiji and New Caledonia) is *Anopheles punctatus* and in Polynesian Islands is *Aedes polynesiensis*.

Wuchereria bancrofti is viviparous or to say ovo-viviparous; its larvae are referred to as microfilariae which/harbour the blood of human beings.

Structure

1. The adult filarial worms (*Wuchereria bancrofti*) are long and hair-like, often creamy-white in colour (Fig 1).

2. The sexes are separate and they show a distinct sexual dimorphism. The males measure 40 mm in length and 0.1 mm in diameter.

3. The females are about 80-100 mm in length and 0.24-0.3 mm in diameter.

4. The tail of the male is curved and there are two spicules of unequal length. It also contains a number of genital papillae and caudal alae.

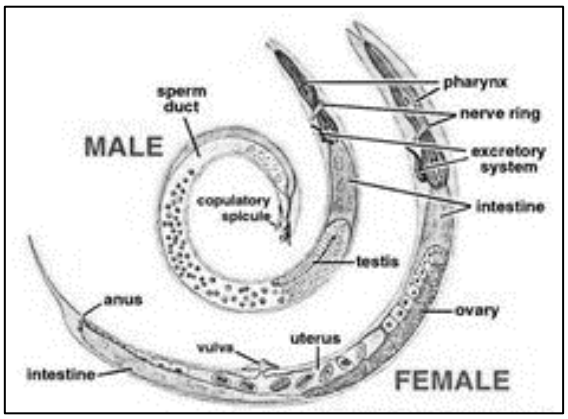

Fig 1: Structure of adult *Wuchereria bancrofti*

5. The body tapers to a slightly swollen head and bearing mouth as a simple pore without lips.

6. The oesophagus is partly muscular and partly glandular.

7. The vulva or genital pore of female opens a little behind the middle and is provided with a pyriform ovijector.

8. The female is ovoviviparous or lays eggs which contain embryos.

The embryo is known as microfilaria (Fig 2). Though they commonly referred to larvae (microfilaria larva) but they should appropriately be termed embryos because their internal organization represents an early developmental stage and they are also not comparable to other nematode larvae.

The microfilariae are very active and can move both with and against the blood stream. They have colourless and transparent bodies with blunt anterior ends and rather pointed tails. A microfilaria measures about 290 pm in length and 6 to 7 pm in diameter.

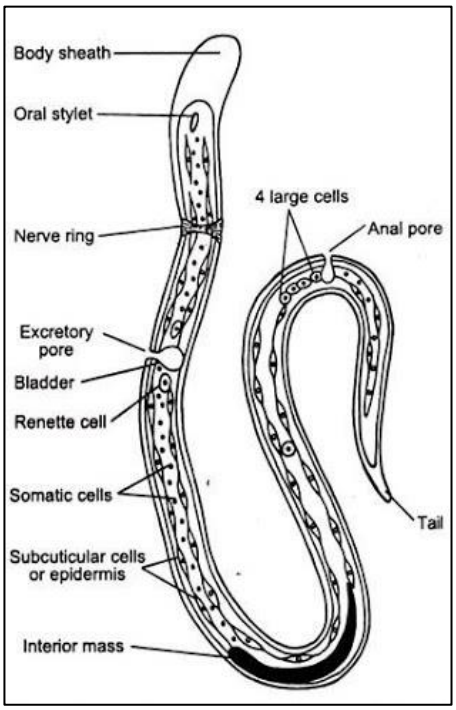

Fig 2: Larval stage (Microfilaria) of *Wuchereria bancrofti*

The body of a microfilaria is covered in a hyaline sheath followed by cuticula being lined by flattened subcuticular cells or epidermis and an inner column of cytoplasm containing nuclei. However, various structures from anterior end downwards are future mouth or oral stylet, nerve ring band, nephridiopore, renette cells and a dark coloured inner mass and four cells of future anus.

The microfilariae do not undergo further development in the human body unless they are taken up by their suitable secondary host (mosquito). If these microfilariae are not sucked up by the mosquito, they die in course of time. Their life span in human body is probably 70 days.

Life Cycle

The life cycle of *Wuchereria bancrofti* (Fig 3) is completed through the two hosts (digenetic), man and mosquito. Man is the definitive host and the adult worms and the live embryos (microfilariae) inhabit in the lymphatic system and blood stream. Mosquito, the species belonging to genus *Culex*, *Aedes* and *Anopheles*, all act as intermediate host. *Culex quinquefasciatus*, *Culex fatigans* and in some places a closely related species, *C. pipiens* play a leading role as vectors of periodic form in different parts of the world.

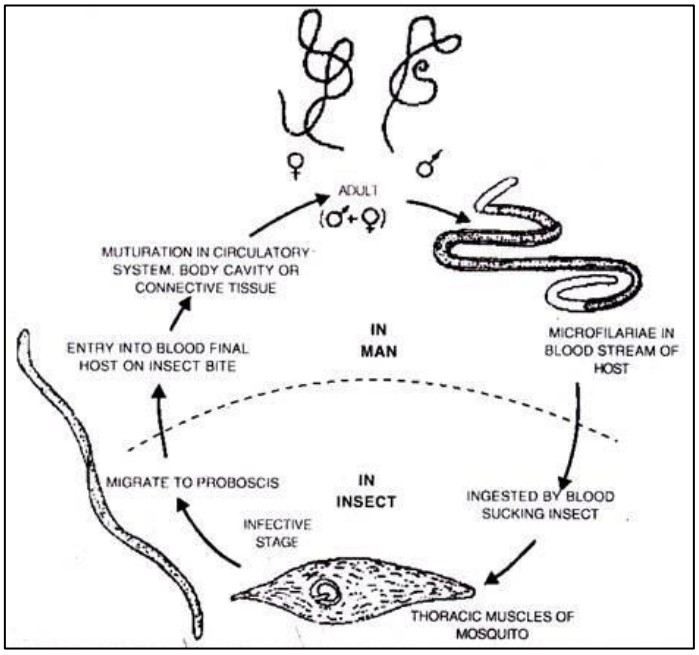

Fig 3: Life cycle of *Wuchereria bancrofti*

Life cycle in man

When both sexes are found in the same afferent lymphatic vessel of human beings, the copulation takes place and each gravid female gives rise to 1000 minute sheathed larvae called microfilaria. These larvae are 127-320 µm long. They can move both with and against the blood stream.

Each larva is colourless, transparent and cylindrical with blunt head. The body is encased in a hyaline sheath and cuticle is thin, striated bearing underneath a single layer of flattened sub-cuticular cells or epidermal cells. The head bears a clear cephalic space and rudiments of adult buccal cavity with oral stylet. Various structures are seen towards backwards next to oral stylet.

These are nerve ring band, renette cells, a darkly stained interior mass, four large cells and future anus. The caudal end is pointed and without nuclei. The body column is provided with somatic cells being interrupted at intervals by cellular and nuclear landmarks.

There is an anterior shiny spot, called rudimentary excretory pore. The relative positions of these landmarks are definite in their body and they cause no trouble to man even if numerous.

The microfilariae are unable to develop further in the human host unless they are ingested by the mosquito. If not sucked up by the mosquito, the larvae will die.

Life cycle in mosquito

When the microfilariae are sucked up by the vector mosquito (*Culex fatigans*) along with the blood meal, the larvae pass to the midgut of the insect and lose their sheaths within 2-6 hours. Then the larvae penetrate the gut wall and migrate to the thoracic muscles. Inside these muscles the organisms become shortened into sausage-shaped forms measuring 124 to 250 µm in length and 10 to 17 µm in breadth.

This stage of development represents the first larval stage in which the first moult occurs. In the first larval stage the tail portion becomes atrophied and possesses the well-defined intestinal tract. Within three to seven days, the larvae grow rapidly, moult once or twice and attain the size which measure about 225-380 µm in length. These represent the second stage larvae.

On the tenth and eleventh day the metamorphosis of the larvae becomes complete inside muscles. The tail portion atrophies and the digestive system, body cavity and genital organs are fully developed. These forms are recognised as third stage filariform larva measuring 1500 to 2000 µm in length and 18-23 µm in breadth.

Then the larva migrates into the proboscis sheath of the mosquito's mouth parts and this stage is infective to man (third stage filariform larvae). It is to be noted that after the second moult third larval stage begins.

Infection to man and development into adult worms

When the infected insect bites the man, the third stage larvae enter the human host through the site of puncture wound on the skin. Then the larvae reach to the lymph vessels via blood stream and settle down in inguinal, scrotal or abdominal lymphatics.
Within five to eighteen months the larvae undergo two additional moults to attain the sexual maturity and start new generation of microfilariae. In the life cycle of *W. bancrofti*, four moults and five stages are recorded of which fifth stage is the adult.

Conditions for development

The development of the infective stage (3rd stage larvae) takes a minimum of 8-10 days but more frequently 2 weeks or more. The optimum conditions are 80 °F and 90% humidity.

Nocturnal and diurnal periodicity

Nocturnal periodicity is the appearance of microfilariae at night in the peripheral circulation of the human host. In the common strain of *W. bancrofti* the nocturnal periodicity is the most common phenomenon but in Polynesian islands of South Pacific except Hawai the microfilariae appear in large numbers in the pulmonary vessels (i.e., blood vessels of the deep tissue of the body) during the day, so it shows a certain degree of diurnal periodicity.

It has been reported that *Aedes pseudoscutellaris*, a mosquito vector of the Pacific strain of *W. bancrofti*, sucks blood during daytime from the body of human's host. During night the microfilariae are found in the peripheral circulation between 10.00 pm to 2.00 am.

Reasons for periodicity:

The exact reasons for nocturnal and diurnal periodicity are not exactly known. In man, the decrease in body temperature, increased O_2 tension, increase in CO_2 pressure, increased body acidity and relaxation of the host during sleep induce the microfilariae to migrate in the peripheral circulation during night. There is also chemotactic between the microfilariae and the saliva of mosquito hosts (vectors) which induces the microfilariae to be more plentiful in the peripheral circulation at night.

Transmission

Adult worms nest in the lymphatic vessels and disrupt the normal function of the lymphatic system. The worms can live for approximately 6–8 years and, during their life time, produce millions of microfilariae (immature larvae) that circulate in the blood.

Mosquitoes are infected with microfilariae by ingesting blood when biting an infected host. Microfilariae mature into infective larvae within the mosquito. When infected mosquitoes bite people, mature parasite larvae are deposited on the skin from where they can enter the body. The larvae then migrate to the lymphatic vessels where they develop into adult worms, thus continuing a cycle of transmission.

Symptoms

The pathogenic effects seen during filariasis are caused by living or dead adult worms.

A light infection does not produce serious effects; it causes filarial fever, headache and mental depression, etc. But, during heavy infection, the accumulated living or dead adult worms block the lymphatic vessels and glands causing lymphatic obstruction so that lymph cannot get back to the circulatory system; resulting the immense enlargement of the affected organs, such as limbs, scrotum, vulva, mammary glands. Ultimately elephantiasis or filariasis or Bancroft's filariasis is produced. It causes filarial fever, headache, mental depression and pain in the swollen area.

Hence, there occurs accumulation of lymph in the affected organs due to which they swell fantastically, a condition called lymphoedema. When they infect lymph nodes then they cause lymphadenitis, in lymph vessels they cause lymphangitis and after infecting epididymis and related areas they cause hydrocele.

However, the affected organs sometimes become enormously enlarged, producing a tumour-like ugly look, this condition is called elephantiasis; the elephantiasis of feet, hands, scrotum, etc., are of common occurrence in the areas where *W. bancrofti* is prevalent.

Prophylaxis

No proper or satisfactory treatment is yet known.

(i) Oral administration of Hetrazan and compounds of antimony and arsenic may be effective by eliminating microfilariae from the blood circulation.

(ii) Administration of Diethylcar- bamazine (DEC) mixed in common salts may be more effective under the National Filaria Control Programme.

(iii) There is no effective drug for elephantiasis.

(iv) Cortisone injection can reduce the swelling.

(v) Large swellings can be removed by surgery.

(vi) Protection from mosquito bites and control of mosquito vectors may be helpful in preliminary level.

Diagonosis

The standard method for diagnosing active infection is the identification of microfilariae in a blood smear by microscopic examination. The microfilariae that cause lymphatic filariasis circulate in the blood at night (called nocturnal periodicity). Blood collection should be done at night to coincide with the appearance of the microfilariae, and a thick smear should be made and stained with Giemsa or hematoxylin and eosin. For increased sensitivity, concentration techniques can be used.

Serologic techniques provide an alternative to microscopic detection of microfilariae for the diagnosis of lymphatic filariasis. Patients with active filarial infection typically have elevated levels of antifilarial IgG_4 in the blood and these can be detected using routine assays.

Because lymphedema may develop many years after infection, lab tests are most likely to be negative with these patients.

- ***Cimex lectularius***

Introduction

The common bed bug (*Cimex lectularius*) has long been a pest – feeding on blood, causing itchy bites and generally irritating their human hosts. The Environmental Protection Agency (EPA), the Centers for Disease Control and Prevention (CDC), and the United States Department of Agriculture (USDA) all consider bed bugs a public health pest. However, unlike most public health pests, bed bugs are not known to transmit or spread disease. They can, however, cause other public health issues, so it's important to pay close attention to preventing and controlling bed bugs.

Distribution

Human dwellings, bird nests, and bat caves are the most suitable habitats for bed bugs because they offer warmth, areas to hide, and hosts on which to feed. Bed bugs are not evenly distributed throughout the environment but are concentrated in harbourages. Within human dwellings, harbourages include cracks and crevices in walls and furniture, behind wallpaper and wood panelling, or under carpeting. Bed bugs are usually only active during the night but will feed during the day when hungry. Bed bugs can be transported on clothing, and in luggage, bedding and furniture. Bed bugs lack appendages that allow them to cling to hair, fur, or feathers, so they are rarely found on hosts.

Habit and Habitat

Bedbugs are found all over the world. They inhabit dark, damp human dwellings such as old houses, buildings, hotels, hostels, rest houses, barracks, carriages and almost anywhere else. They live in cracks in the walls and floor, in crevices in the beds and furniture, under mattresses, carpets and wall

paper and in similar places. The thin bodies of bedbugs are well adapted to life in narrow spaces or crevices.

Bedbugs are nocturnal, but often come out during the day. They are gregarious insects. These are sanguinivorous ectoparasites. They are strongly attracted by the warmth and the odour of the body. They are incapable of flight but they migrate from one house to another along the walls, pipes or drains.

They are carried from one place to another on clothing, in luggage or furniture. If not allowed to reach the beds by placing their legs in water troughs or other barriers, they climb up the wall and move along the ceiling to drop down from there on the beds. They usually suck human blood, but also attack warm blooded animals such as birds (domestic fowl) and mammals (mice, rats, rabbits, cats and dogs).

They suck human blood while the man is sleeping, after which they quickly run away. Their gregarious habit results in great discomfort to mankind. Bedbugs can survive without food for several months or a year and even longer. The starving bedbugs may feed upon birds, rats, mice and rabbits and may also resort to cannibalism.

Their retiring habits coupled with their power to fast for long periods make their eradication difficult. Bedbugs give out a peculiar kind of odour or foul smell due to the presence of secretion of stink gland. They are oviparous and undergo gradual metamorphosis.

Structure

The morphology of bed bug is described below:

Adult bed bugs, in general (Fig 4,5) are:

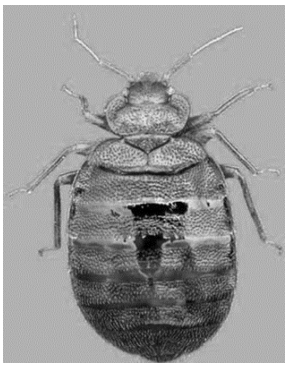

Fig 4: Dorsal view of an adult bed bug, *Cimex lectularius*

- about the size of an apple seed (average 5.5 mm and the width 2.5 mm);
- long and brown, with a flat, oval-shaped body (if not fed recently);
- balloon-like, reddish-brown, and more elongated (if fed recently);
- Females are larger than males.
- A “true bug” (characteristics of true bugs include a beak with three segments; antenna that have four parts; the first segment is shorter than other segments. The three segments closest to the antenna tip are thin and elongated; wings that are not used for flying; and short, golden-coloured hairs)
- The head is short, broad and pointed at the tip and had a pair of prominent red or black compound eyes resembling raspberries.

- Mouthparts or labium are three-segmented and specialized in piercing and sucking and are located on the ventral side of the head. The tip of the labium extends to the level of the first pair of legs.
- The thorax also is three-segmented.
- Smelly, with a "musty-sweetish" odour produced through glands on the lower side of the body.

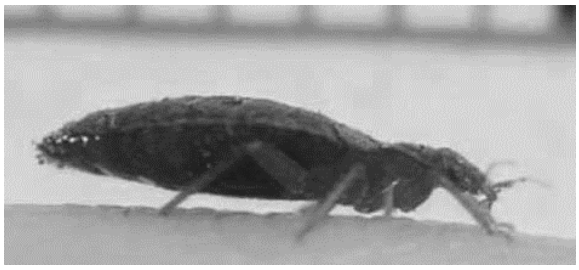

Fig 5: Lateral view of an adult bed bug, *Cimex lectularius*

Young bed bugs (also called nymphs), in general, are (Fig 6):

- smaller, translucent or whitish-yellow in colour; and
- If not recently fed, can be nearly invisible to the naked eye because of colouring and size.

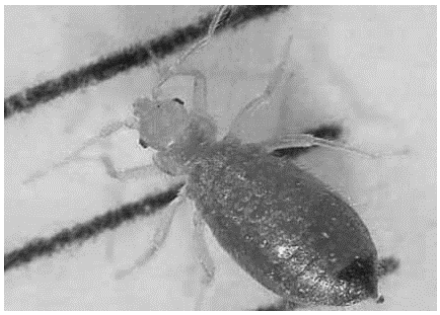

Fig 6: Nymph of the bed bug, *Cimex lectularius*

Bed bug eggs, in general, are:

- tiny, the size of a pinhead;
- pearl-white in colour; and
- Marked by an eye spot if more than five days old.

Life Cycle

Before laying eggs, the female bug feeds on blood-meal and mates with the male bug. Life history of bedbug exhibits gradual metamorphosis and comprises three stages: egg, nymph and adult (Fig).

(i) Mating:

In bedbugs, the mating or copulation is quite interesting. While mating or copulating, the male bug takes up a position diagonally across the body of female bug and introduces its penis into the notch or cleft of the organ of Berlese to transfer the spermatophore. The spermatozoa bore through the wall of organ of Berlese and reach the ovary to fertilize the eggs. Thus, fertilisation is internal.

(ii) Eggs:

The female lays about 200 to 500 eggs, singly or in batches, 2 or 3 eggs per day, in cracks and crevices of cots and furniture, in holes, under mattresses and similar other places. The eggs of

bedbugs are pearly white oval or cylindrical objects, furnished with a little cap-like lid at one end which is slightly curved to one side.

The end possessing the cap-like lid bears a micropyle. The eggs are about 1.00 mm in length and are laid singly or in small batches. The eggs are laid throughout the year in warm countries.

(iii) Nymph:

The eggs hatch in from 6 to 10 days during warm weather but take a longer period during cold weather as their development is retarded by cold. The young bugs or nymphs come out by pushing off the lids of the eggs. The nymphs are very small, about 1.00 to 1.50 mm long, flat, active, delicate, semi-transparent creatures and are pale in colour.

They resemble the parents in general appearance except being smaller and paler and possessing comparatively thicker antennae and stouter legs. After a few hours, the nymphs are able to pierce the skin of man and suck the blood, and if undisturbed they feed to repletion in about 3 or 4 minutes.

They may take a meal three or four times of their body weight and become globular and bright red. They need shelter but no more food till they moult into the second stage. After their first hearty meal they have a much more robust appearance, and grow rapidly. They feed on human blood and moult five times to become the adult.

After each moult, the nymphs become slightly larger and darker (Fig 7). If man is not available for feeding, they feed on blood from the older bugs. The nymphs can survive without feeding for 3 or 4 months during which period they do not moult. After five moults they become adult taking about 7 to 24 weeks in all. The entire life history takes about 2 months in warm weather and about 6 months during winter in cold regions.

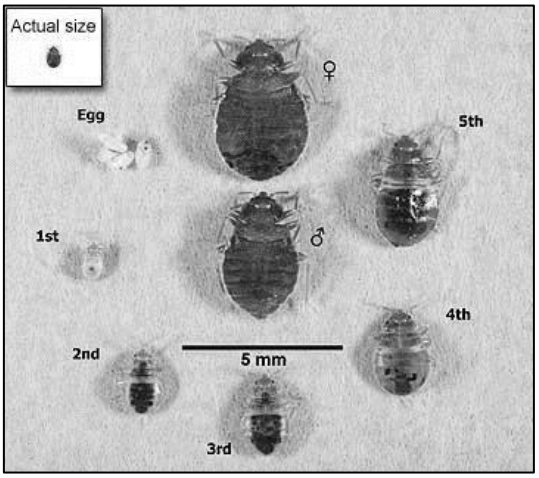

Fig 7: Life cycle and different nymph stages of *Cimex lectularius*

Symptom

Cimex lectularius, the bed bug has been suspected of being the cause of many human diseases, but this has not been proved. After identification of bedbug bites, skin and infectious transmissible diseases are the 2 main medical concerns of human contact with the bedbugs. Hosts are usually bitten at night. Small, flat, or raised bumps on the skin are the most common sign; redness, swelling, and itching commonly occur. If scratched, the bite areas can become infected. Bedbug saliva contains anesthetic compounds, bites are painless and usually not felt until several hours later. Other compounds are also injected: anticoagulant factors (eg, factor-X inhibitor), vasodilatory compounds

(such as nitric oxide), and proteolytic enzymes (eg, apyrase), which are all substances that participate in the ensuing local hypersensitivity reactions.

The typical skin lesion is a pruritic erythematous maculopapule, 5 mm to 2 cm in diameter, with a central hemorrhagic crust or vesicle at the bite site, similar to arthropod bites. Atypical forms vary from asymptomatic or pauci-symptomatic to purpuric, vesicular, and bullous lesions. The bedbug-bite distribution frequently follows a line or curve (Figure 8A and 8B). Lesion numbers range from several to many, depending on habitat-infestation intensity, and are preferentially located in unclothed zones (Figure 8*C*). Sometimes, the eruption mimics urticaria (Figure 8D).

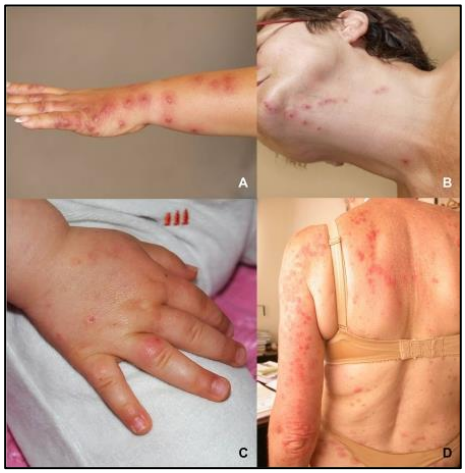

Fig 8: Presentation of bedbug (*Cimex lecturarius*) bites: forms vary from asymptomatic or pauci-symptomatic to purpuric, vesicular, and bullous lesions. The typical skin lesion is a pruritic erythematous maculopapule that is 5 mm to 2 cm in diameter with a central hemorrhagic crust or vesicle at the bite site, similar to other arthropod bites (*A*). A series of bites in a line is characteristic of bedbug bites (*B*). Lesion numbers range from a few to numerous, depending on habitat-infestation intensity, and are preferentially located in unclothed zones (*C*). In some cases, the eruption mimics urticaria (*D*).

In the gut of bed bugs are anti-bacterial substances which do not permit bacteria to live for long. Cimex may carry and transmit germs of plague and relapsing fever, this is only for short periods.

Prophylaxis

1. Typically, no treatment is required for bedbug bites.
2. If itching is severe or if an allergic reaction to the bites occurs, topical steroid creams or oral antihistamines may be used for symptom relief.
3. Secondary bacterial infections that develop over heavily scratched areas may require antibiotics.
4. Anything that relieves and controls itching, such as cool compresses, oatmeal baths, or a paste made of baking soda and water.

Diagnosis

If you suspect that you're being bitten by bedbugs, immediately inspect your home for the insects. Thoroughly examine crevices in walls, mattresses and furniture. You may need to perform your inspection at night when bedbugs are active.

Look for these signs:

Dark specks. Typically found along mattress seams, these specks are bedbug excrement.

Empty exoskeletons. Bedbugs molt five times before becoming adults. These empty skins are pale yellow.
Rusty or reddish stains. You may find small smears of blood on your bed sheets where you accidentally crushed a bedbug.

- ***Xenopsylla cheopis***

Introduction

The Oriental rat flea (*Xenopsylla cheopis*), also known as the tropical rat flea, is a parasite of rodents, primarily of the genus *Rattus*, and is a primary vector for bubonic plague and murine typhus. This occurs when the flea has fed on an infected rodent and bites a human, although this flea can live on any warm blooded mammal.

Geographic distribution

Xenopsylla cheopis usually inhabits tropical and subtropical habitats, although it has been reported in the temperate zone as well. *Xenopsylla cheopis* is rarely found in cold areas since it requires a tropical/subtropical climate to pupate. Fleas are prevalent in many major cities. Species of *Rattus* typically found in city sewer systems and other human related habitats are excellent hosts for *X. cheopis*. Seaports and other rat-infested areas are also common habitats for *X. cheopis*.

Fleas are nidiculous parasites; they live in the host's nest. Clothing, beds and couches make perfect homes for many of these fleas. Fleas only attach to the host while they are sucking blood; at other times they are free-living in the host's nest.

Habitat Regions: temperate tropical terrestrial
Terrestrial Biomes: desert or dune savannah or grassland chaparral forest rainforest scrub Forest Mountains
Other Habitat: Urban, Suburban, and Agricultural

Habit and habitat

Adults of both sexes of *Xenopsylla cheopis* feed on blood. They bite *Rattus rattus* (Black Rat) and other mammals, including humans. *Xenopsylla cheopis* obtains the host's blood through a set of external mouthparts, which consist of the following maxillary lacunae and an epipharynx. The purpose of each structure is to aid in the sucking up of blood. After biting, the fleas suck blood from a pool (telmophagy), unlike some other insects like mosquitoes that feed directly from the blood vessel (solenophagy).

Piercing of the host's skin is achieved by the back and forth action of the maxillary laciniae. After the skin is cut the epipharynx enters the wound and injects salvia. Saliva contains special chemicals, which keep the host's blood from coagulating. A canal formed by the maxillary laciniae and the epipharnyx then sucks up blood. Further down the gut a specialized organ called the proventriclus then breaks down blood cells enabling the *X. cheopis* to digest the blood meal. The average capacity of *Xenopsylla cheopis* is 0.5 cubic millimeters.

The larvae of *X. cheopis* have mandibles, which they use to feed on detritus and the faeces of the adult fleas, which are found in the nests of hosts.

Structure

Morphological structure of adult *Xenopsylla cheopis* is described below (Fig 9)

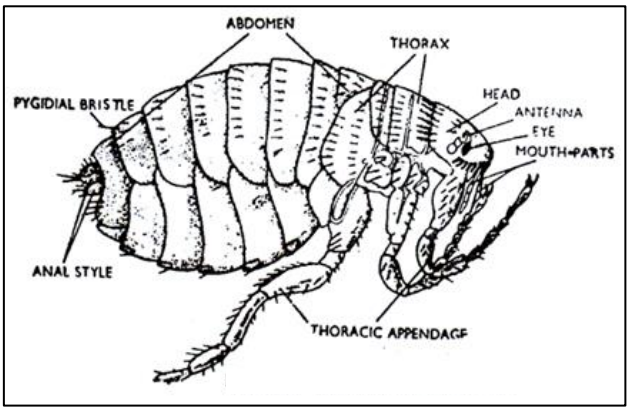

Fig 9: Morphology of *Xenopsylla cheopis*

1. It is commonly known as (Oriental rat flea or Pissu), and is common in tropics.

2. It is an ectoparasite on rats and other such mammals and acts as disease vector for plague.

3. Body is laterally compressed and maybe divided into head thorax and abdomen, which may not be demarcated clearly.

4. The head bears a small 3 to 4 jointed antennae a pair of simple eyes and piercing & sucking or siphoning mouthparts.

5. Thorax bears three pairs of long, jointed and clawed legs for hopping but no wings.

6. Abdomen is swollen in middle and has 8 segments and a pair of anal styles.

7. The whole body is covered over with bristles on the dorsal as well as lateral sides.

Life Cycle

Fleas are holometabolous, which means they go through four life-cycle stages: egg (embryo), larva, pupa, and adult (imago) (Fig 10).

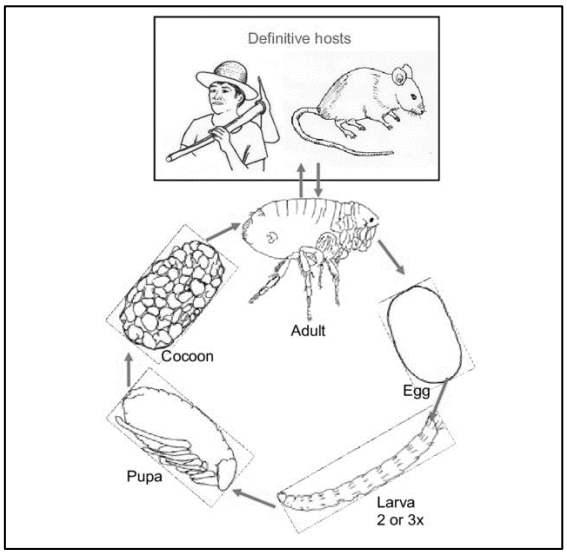

Fig 10: Life cycle of *Xenopsylla cheopis*

No information is available on the mating systems of these fleas.

After copulating with a male the female is ready to lay her eggs. She does this at frequent intervals while feeding. *Xenopsylla cheopis* prefers temperatures of 65 to 80°F with about 70% humidity for egg laying. Higher or lower temperatures inhibit females from laying their eggs. Eggs usually do not hatch on the hosts, rather on their nests since fleas are nidiculous parasites (they live on host's nests). These fleas breed year round, as long as the temperature and humidity favour egg-laying. *Xenopsylla cheopis* is distinct from other fleas in that it has a very large egg. Studies demonstrate that eggs of *X. cheopis* obtain extra nutrients from their mother, hence explaining the abnormally large egg. Once eggs are laid, however, they receive no further support from their parents.

They hatch into a larva that looks very similar to a worm and is about 2mm long. It only has a small body and a mouth part. At this stage, the flea does not drink blood; instead it eats dead skin cells, flea droppings, and other smaller parasites lying around them in the dust. When the larva is mature it makes a silken cocoon around itself and pupates. The flea remains a pupa from one week to six months changing in a process called metamorphosis. When the flea emerges, it begins the final cycle, called the adult stage. A flea can now suck blood from host and mate with other fleas. A single female flea can mate once and lay eggs every day with up to 50 eggs per day.

Transmission

This species can act as a vector for plague, *Yersinia pestis*. *Yersinia pestis* is transmitted in epidemics from rats to humans via the rat flea, *Xenopsylla cheopis*. Numerous rodents and other mammals serve as reservoirs of *Y. pestis*, some of which have been responsible for cases of human plague.

Naturally acquired plague in people occurs as a result of human intrusion into the zoonotic cycle during or following an epizootic, or by the entry of sylvatic rodents or their infected fleas into man's habitat with infection in commensal rodents and their fleas; this may result in the development of a domestic rat epizootic and epidemic plague. Domestic pets, particularly house cats and dogs, may carry plague infected wild rodent fleas into homes, and cats may occasionally transmit infection by their bites or scratches; cats develop plague abscesses that have been a source of infection to veterinarians.

The most frequent source of exposure that results in human disease worldwide has been the bite of infected fleas (especially *Xenopsylla cheopis*, the oriental rat flea). Other important sources include the handling of tissues of infected animals, especially rodents and rabbits but also carnivores; rarely airborne droplets from human patients or household cats with plague pharyngitis or pneumonia; or careless manipulation of laboratory cultures.

Symptom

- Bubonic plague: enlarged, tender lymph nodes, fever, chills and prostration.
- Septicaemia plague: fever, chills, prostration, abdominal pain, shock and bleeding into skin and other organs.
- Pneumonic plague: fever, chills, cough and difficulty breathing; rapid shock and death if not treated early.

Prophylaxis

- People with the plague need to be treated right away. If treatment is not received within 24 hours of when the first symptoms occur, the risk for death increases.

- Admitted to a hospital.
- Receive powerful antibiotics, such as: Gentamicin, Doxycycline (Monodox, Vibramycin, others), Ciprofloxacin (Cipro), Levofloxacin, Moxifloxacin (Avelox), Chloramphenicol
- Oxygen, intravenous fluids, and respiratory support are usually also needed.
- People with pneumonic plague must be kept away from caregivers and other patients. People who have had contact with anyone infected by pneumonic plague should be watched carefully and given antibiotics as a preventive measure.

Diagnosis

If your doctor suspects plague, he or she may look for the *Yersinia pestis* bacteria in samples taken from your:

- **Buboes.** If you have the swollen lymph nodes (buboes) typical of bubonic plague, your doctor may use a needle to take a fluid sample from them (aspiration).
- **Blood.** You'll generally have Yersinia pestis bacteria present in your bloodstream only if you have septicemic plague.
- **Lungs.** To check for pneumonic plague, your doctor will take mucus (sputum) or fluid from your airways using a thin, flexible tube inserted through your nose or mouth and down your throat (endoscopy).

Probable questions:

1. Name the causative agents of filariasis.
2. Name the secondary host of filariasis of India and Pacific Island.
3. Describe the structure if microfilaria with diagram.
4. Write short note on diagnosis of filariasis.
5. State the morphology of *Cimex lecturarius* with diagram.
6. How can you identify bed bug bites?
7. How can you treat the bed bug bites?
8. Discuss the pathobiology and prophylaxis of *Cimex lecturarius*.
9. Describe the morphology of *Xenopsylla cheopis* with diagram.
10. Briefly discuss the pathobiology of *Xenopsylla cheopis.*

Suggested readings:

1. Frishman A. 2000. Bed Bug basics and control measures. Pest Control 68: 24.
2. Snetsinger R. 1997. Bed bugs & other bugs, pp. 393-425. *In* Mallis A, Hedges SA [eds.], Handbook of Pest Control, 8th ed. Franzak & Foster Co., Cleveland, Ohio.
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5. Paniker, C.K.J., Ghosh, S. [Ed} (2013). Paniker's Text Book of Medical Parasitology. Jaypee, New Delhi.

DISCLAIMER: This Self Learning Material (SLM) has been compiled from various authentic books, Journals articles, e-journals and other web sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-IV

SOFT CORE THEORY PAPER

MEDICAL EMBRYOLOGY (ZST-409)

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

W.B. INDIA

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Director
Prof. Manas Mohan Adhikary
Directorate of Open and Distance Learning
University of Kalyani

SOFT CORE THEORY PAPER (ZST -409)

MEDICAL EMBRYOLOGY

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZST - 409 (MEDICAL EMBRYOLOGY)	I	Medical implications : Infertility- Diagnostic infertility, causes of infertility.	1.0	1	1	
	II	Assisted Reproductive Technologies : Sperm and ova bank; Artificial Insemination donor (AID); in vitro fertilization (IVF), procedures, variations of IVF, Success rates and complications; Gamete Intrafallopian transfer (GIFT), Intracytoplasmic sperm Injection (ICSI), Surrogate mothers.		1	1	
	III	Genetic errors of human development- Down syndrome, Fragile X syndrome. Techniques used in Medical Embryology : i) Amniocentesis ii) Chorionic villus sampling iii) Ultrasonography.		1	1	
	IV	Future of medicine: Differentiation therapy, gene therapy (Ex Vivo and In vivo), germ line gene therapy.		1	1	

Unit-I

Medical implications : Infertility- Diagnostic infertility, causes of infertility

Objective: In this unit we will discuss different aspects of male and female infertility including causes of infertility and how it can be diagnosed.

Infertility:

Infertility is a term that is defined as the inability of couples to become pregnant after one year of regular intercourse without contraception, or 6 months if a woman is 35 years or older. Infertility can occur in both females and males, and there are many causes. In order to get pregnant, a woman's body must go through three main steps: ovulation, fertilization, and implantation. In ovulation, an egg is released from one of her ovaries, which then travels down to one of the fallopian tubes and into the uterus. The egg must then be fertilized by the sperm during the process of fertilization. Finally, in implantation, the fertilized egg implants itself in the uterine wall. When there are problems in any of these steps, infertility can be diagnosed.

Types of Infertility: Infertility can be primary or secondary.

Primary infertility is when a couple has not conceived after trying for at least 12 months without using birth control

Secondary infertility is when they have previously conceived but are no longer able to.

Causes of Infertility:

The causes of infertility may be physical, congenital, disease, drug, immunological or even psychological. In India, when a couple is childless, the female is usually blamed. But more often, the males are detected to be responsible. However, now, specialized health care units known as infertility clinics are available. They could identify the cause of infertility and take up treatment to remove the disorder.

A. Male Infertility:

Infertility in males may be due to the following causes:

a. Azoospermia:

Absence of sperms in the semen is known as azoospermia. This may occur because of lack of sperm production or because of blocked tubes which does not permit the sperms to appear in the semen. Blockage can occur due to an infection or injury.

Failure of the ejaculation mechanism is another possible reason of azoospermia. Failure to produce sperms may result because of injury to the testes or as a result of infection such as mumps virus or due to hormonal reasons (Fig. 3).

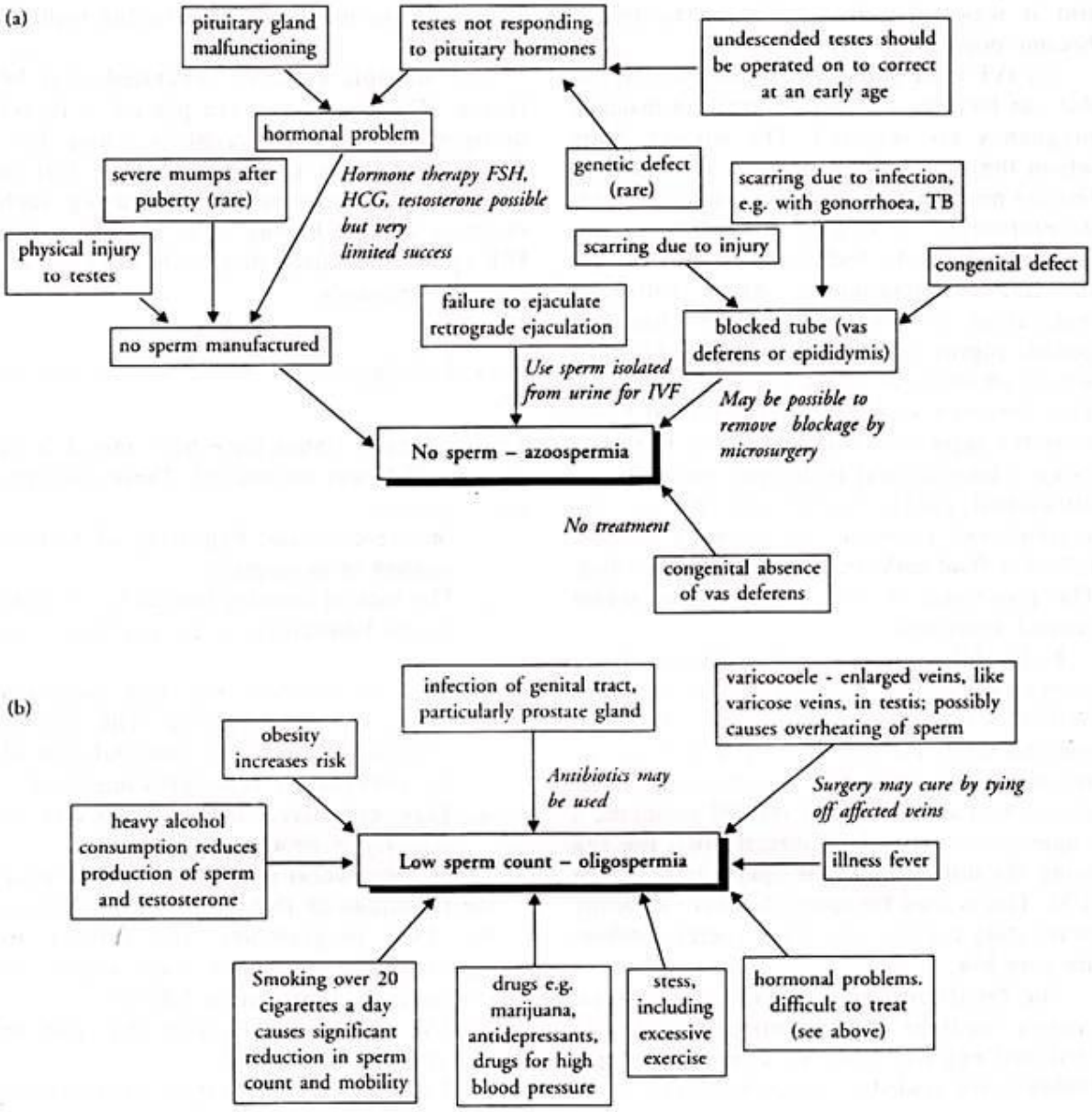


Fig. 3 The causes and treatment of azoospermia and oligospermia.

b. Oligospermia:

Low sperm count is known as oligospermia. More than 90% males suffer from infertility due to low sperm count. The reasons of oligospermia is summarised in Fig. 3.

c. Abnormal Sperms:

Abnormal sperms may possess two heads, or no tail or may have abnormal shapes (Fig. 4). The reasons are not known and may be because of hormonal malfunctions.

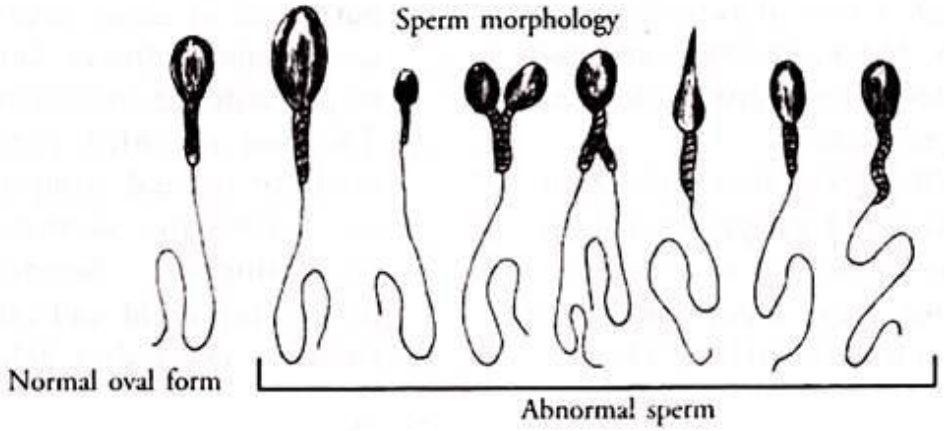


Fig. 4 Semen analysis.

d. Autoimmunity:

In some males, the immune system may attack the sperms and reduce the sperm numbers. Treatment is not usually possible.

e. Impotence and Premature Ejaculation:

The inability to achieve an erection of the penis is known as impotence. Psychological counselling may help in some cases. Premature ejaculation is a condition where the man releases the semen even before penetration into the vagina. This condition is treatable with psychological treatment.

f. Immotile cilia:

Absence of tail in sperm makes it immotile. Hence, sperms cannot move from vagina to upper portions of genital tract of female.

g. Absence of Y-chromosome:

Sometimes, deletion of Y-chromosomes in primordial germ cells leads to sperm production without Y-chromosome. Such sperms cannot form viable zygole.

h. Tubular blockage:

Blockage of vasa deferentia and vasa efferentia stops sperm transport.

i. Antisperm antibodies:

Such antibodies are IgG, IgM and IgA. Sometimes IgG is found in cervical mucous, serum and semen.

j. High scrotal temperature:

Due to development of dilated veins in testis (varicocela) scrotal temperature is raised and sperm production is minimized leading to oligospermia.

k. Low fructose content and high prostaglandin in seminal fluid lead to sperm destruction.

l. Vasectomy leads to irreversible infertility in males.

Other causes of male sterility may include:

a. Genetic factors: A man should have an X and Y chromosome. If he has two X chromosomes and one Y chromosome, as in Klinefelter's syndrome, the testicles will develop abnormally and there will be low testosterone and a low sperm count or no sperm.

b. Mumps: If this occurs after puberty, inflammation of the testicles may affect sperm production.

c. Hypospadias: The urethral opening is under the penis, instead of its tip. This abnormality is usually surgically corrected in infancy. If the correction is not done, it may be harder for the sperm to get to the female's cervix. Hypospadias affects about 1 in every 500 newborn boys.

d. Cystic fibrosis: This is a chronic disease that results in the creation of a sticky mucus. This mucus mainly affects the lungs, but males may also have a missing or obstructed vas deferens. The vas deferens carries sperm from the epididymis to the ejaculatory duct and the urethra.

e. Radiation therapy: This can impair sperm production. The severity usually depends on how near to the testicles the radiation was aimed.

f. Some diseases: Conditions that are sometimes linked to lower fertility in males are anaemia, Cushing's syndrome, diabetes, and thyroid disease.

Some medications increase the risk of fertility problems in men.

a. Sulfasalazine: This anti-inflammatory drug can significantly lower a man's sperm count. It is often prescribed for Crohn's disease or rheumatoid arthritis. Sperm count often returns to normal after stopping the medication.

b. Anabolic steroids: Popular with bodybuilders and athletes, long-term use can seriously reduce sperm count and mobility.

c. Chemotherapy: Some types may significantly reduce sperm count.

d. Illegal drugs: Consumption of marijuana and cocaine can lower the sperm count.

e. Age: Male fertility starts to fall after 40 years.

f. Exposure to chemicals: Pesticides, for example, may increase the risk.

g. Excess alcohol consumption: This may lower male fertility. Moderate alcohol consumption has not been shown to lower fertility in most men, but it may affect those who already have a low sperm count.

h. Mental stress: Stress can be a factor, especially if it leads to reduced sexual activity.

B. Female Infertility:

A woman may be infertile due to several causes.

Some important reasons are as follows:

a. Failure to Ovulate: Failure to ovulate is one common cause of infertility in females. This is because the pituitary or hypothalamus fails to produce the FSH which is required for follicle

development or LH required for release of the egg from the ovary. It may also be because the ovaries fail to produce oestrogen or progesterone. Hormonal imbalances may be corrected by administering synthetic hormones to the affected individual.

The most commonly used drug is Clomiphene, a synthetic oestrogen like drug which stimulates ovulation. Tamoxifen is another drug used. These pills are taken orally for five days soon after the menstrual cycle starts. Injection of HCG, which is chemically similar to LH is given at the middle of the cycle to stimulate ovulation. 'Fertility drugs' which contains FSH and LH or only FSH is also used. But these have the danger of multiple egg release and consequently multiple pregnancies. Advance techniques include small implants in the upper arm which releases small amounts of GnRH mimicking the activity of the hypothalamus.

b. Damage to Oviducts:

The fallopian tubes may be blocked or narrowed in some women. This interferes with the movement of the eggs and fertilisation. This can be treated by laser surgery.

c. Damage to Uterus:

In about 5-10% cases, infertility problems are due to a damaged uterus. The uterus is unable to maintain pregnancy, i.e., the fertilised zygote does not get implanted. Sometimes large non-malignant tumours called fibroids or smaller growths known as polyps which grow in the walls of the uterus can cause infertility. These can be surgically removed. IUCD or PID also causes inflammation in the uterus and cause problems. This can be treated by using antibiotics. Adhesion in the uterus, i.e. sticking of parts of the uterus which occurs as a result of an abortion is another reason for infertility.

d. Damage to the Cervix:

The cervix is the neck of the uterus. The cervix may become damaged because of the abortion or difficult birth. A narrow cervix may interfere with sperm movement.

e. Antibodies to Sperm:

In some rare cases, women may produce antibodies against sperms. These are found in the cervix, uterus and oviducts. These may be treated using immunosuppressant drugs, but IVF is a better method of treatment.

f. Ovarian problem:

There may not be normal ovulation in ovary. Sometimes there is failure of corpus luteum formation.

g. Hormonal cause:

Decreased level of FSH and LH, drug induced ovulation may not allow fertilization and development of the foetus.

h. Uterine factor:

Unfavourable endometrium for implantation, chronic endometritis, fibroid uterus etc. may be the cause of infertility.

i. Cervical factor: In effective sperm penetration, chronic cervicitis, presence of anti sperm antibody and elongation of cervix may be the cause of infertility.

j. Fimbriae:

Fimbriae of Fallopian tube may not pick up secondary oocyte from ovary.

k. Dyspareunia:

Painful sexual intercourse experienced by female may be another cause of infertility.

l. Macrophages:

Increased sperm phagocytosis by macrophages may be the cause of infertility.

m. Miscarriage:

Early miscarriage before complete development of foetus due to various gyaenic problems may be also the reason of infertility.

n. Tubectomy:

Like vasectomy in males, tubectomy in females causes permanent infertility.

Other causes of female sterility may include:

a. Age: The ability to conceive starts to fall around the age of 32 years.

b. Smoking: Smoking significantly increases the risk of infertility in both men and women, and it may undermine the effects of fertility treatment. Smoking during pregnancy increases the chance of pregnancy loss. Passive smoking has also been linked to lower fertility.

c. Alcohol: Any amount of alcohol consumption can affect the chances of conceiving.

d. Being obese or overweight: This can increase the risk of infertility in women as well as men.

e. Eating disorders: If an eating disorder leads to serious weight loss, fertility problems may arise.

f. Diet: A lack of folic acid, iron, zinc, and vitamin B-12 can affect fertility. Women who are at risk, including those on a vegan diet, should ask the doctor about supplements.

g. Exercise: Both too much and too little exercise can lead to fertility problems.

h. Sexually transmitted infections (STIs): Chlamydia can damage the fallopian tubes in a woman and cause inflammation in a man's scrotum. Some other STIs may also cause infertility.

i. Exposure to some chemicals: Some pesticides, herbicides, metals, such as lead, and solvents have been linked to fertility problems in both men and women. A mouse study has suggested that ingredients in some household detergents may reduce fertility.

j. Mental stress: This may affect female ovulation and male sperm production and can lead to reduced sexual activity.

Some medical conditions can affect fertility:

Ovulation disorders appear to be the most common cause of infertility in women. Ovulation is the monthly release of an egg. The eggs may never be released or they may only be released in some cycles.

Ovulation disorders can be due to:

a. Premature ovarian failure: The ovaries stop working before the age of 40 years.

b. Polycystic ovary syndrome (PCOS): The ovaries function abnormally and ovulation may not occur.

c. Hyperprolactinemia: If prolactin levels are high, and the woman is not pregnant or breastfeeding, it may affect ovulation and fertility.

d. Poor egg quality: Eggs that are damaged or develop genetic abnormalities cannot sustain a pregnancy. The older a woman is, the higher the risk.

e. Thyroid problems: An overactive or underactive thyroid gland can lead to a hormonal imbalance.

f. Chronic conditions: These include AIDS or cancer.

Problems in the uterus or fallopian tubes can prevent the egg from traveling from the ovary to the uterus, or womb. If the egg does not travel, it can be harder to conceive naturally.

Causes include:

a. Surgery: Pelvic surgery can sometimes cause scarring or damage to the fallopian tubes. Cervical surgery can sometimes cause scarring or shortening of the cervix. The cervix is the neck of the uterus.

b. Submucosal fibroids: Benign or non-cancerous tumours occur in the muscular wall of the uterus. They can interfere with implantation or block the fallopian tube, preventing sperm from fertilizing the egg. Large submucosal uterine fibroids may make the uterus' cavity bigger, increasing the distance the sperm has to travel.

c. Endometriosis: Cells that normally occur within the lining of the uterus start growing elsewhere in the body.

d. Previous sterilization treatment: In women who have chosen to have their fallopian tubes blocked, the process can be reversed, but the chances of becoming fertile again are not high.

Diagnosis of Infertility:

Most people will visit a physician if there is no pregnancy after 12 months of trying. If the woman is aged over 35 years, the couple may wish to see a doctor earlier, because fertility testing can take time, and female fertility starts to drop when a woman is in her 30s. A doctor can give advice and carry out some preliminary assessments. It is better for a couple to see the doctor together. The doctor may ask about the couple's sexual habits and make recommendations regarding these. Tests and trials are available, but testing does not always reveal a specific cause.

Infertility tests for men:

The doctor will ask the man about his medical history, medications, and sexual habits and carry out a physical examination. The testicles will be checked for lumps or deformities, and the shape and structure of the penis will be examined for abnormalities.

a. Semen analysis: A sample may be taken to test for sperm concentration, motility, colour, quality, any infections, and whether any blood is present. Sperm counts can fluctuate, so that several samples may be necessary.

b. Blood test: The lab will test for levels of testosterone and other hormones.

c. Ultrasound: This may reveal issues such as ejaculatory duct obstruction or retrograde ejaculation.

d. Chlamydia test: Chlamydia can affect fertility, but antibiotics can treat it.

Infertility tests for women:

A woman will undergo a general physical examination, and the doctor will ask about her medical history, medications, menstruation cycle, and sexual habits.

She will also undergo a gynaecologic examination and a number of tests:

a. Blood test: This can assess hormone levels and whether a woman is ovulating.

b. Hysterosalpingography: Fluid is injected into the woman's uterus and X-rays are taken to determine whether the fluid travels properly out of the uterus and into the fallopian tubes. If a blockage is present, surgery may be necessary.

c. Laparoscopy: A thin, flexible tube with a camera at the end is inserted into the abdomen and pelvis, allowing a doctor to look at the fallopian tubes, uterus, and ovaries. This can reveal

signs of endometriosis, scarring, blockages, and some irregularities of the uterus and fallopian tubes.

Other tests include:

a. ovarian reserve testing, to find out how effective the eggs are after ovulation

b. genetic testing, to see if a genetic abnormality is interfering with fertility

c. pelvic ultrasound, to produce an image of the uterus, fallopian tubes, and ovaries

d. Chlamydia test, which may indicate the need for antibiotic treatment

e. thyroid function test, as this may affect the hormonal balance.

Ovarian hyperstimulation syndrome:

The ovaries can swell, leak excess fluid into the body, and produce too many follicles, the small fluid sacs in which an egg develops.

Ovarian hyperstimulation syndrome (OHSS) usually results from taking medications to stimulate the ovaries, such as clomifene and gonadotrophins. It can also develop after IVF.

Symptoms include:

- bloating
- constipation
- dark urine
- diarrhoea
- nausea
- abdominal pain
- vomiting

They are usually mild and easy to treat. Rarely, a blood clot may develop in an artery or vein, liver or kidney problems can arise, and respiratory distress may develop. In severe cases, OHSS can be fatal.

Ectopic pregnancy

This is when a fertilized egg implants outside the womb, usually in a fallopian tube. If it stays in there, complications can develop, such as the rupture of the fallopian tube. This pregnancy has no chance of continuing.

Immediate surgery is needed and, sadly, the tube on that side will be lost. However, future pregnancy is possible with the other ovary and tube. Women receiving fertility treatment have a slightly higher risk of an ectopic pregnancy. An ultrasound scan can detect an ectopic pregnancy.

Treatment:

Treatment will depend on many factors, including the age of the person who wishes to conceive, how long the infertility has lasted, personal preferences, and their general state of health.

Frequency of intercourse

The couple may be advised to have sexual intercourse more often around the time of ovulation. Sperm can survive inside the female for up to 5 days, while an egg can be fertilized for up to 1 day after ovulation. In theory, it is possible to conceive on any of these 6 days that occur before and during ovulation. However, a survey has suggested that the 3 days most likely to offer a fertile window are the 2 days before ovulation plus the 1 day of ovulation. Some suggest that the number of times a couple has intercourse should be reduced to increase sperm supply, but this is unlikely to make a difference.

Fertility treatments for men: Treatment will depend on the underlying cause of the infertility.

a. Erectile dysfunction or premature ejaculation: Medication, behavioural approaches, or both may help improve fertility.

b. Varicocele: Surgically removing a varicose vein in the scrotum may help.

c. Blockage of the ejaculatory duct: Sperm can be extracted directly from the testicles and injected into an egg in the laboratory.

d. Retrograde ejaculation: Sperm can be taken directly from the bladder and injected into an egg in the laboratory.

e. Surgery for epididymal blockage: A blocked epididymis can be surgically repaired. The epididymis is a coil-like structure in the testicles which helps store and transport sperm. If the epididymis is blocked, sperm may not be ejaculated properly.

Fertility treatments for women: Fertility drugs might be prescribed to regulate or induce ovulation. They include:

a. Clomifene (Clomid, Serophene): This encourages ovulation in those who ovulate either irregularly or not at all, because of PCOS or another disorder. It makes the pituitary gland release more follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

b. Metformin (Glucophage): If Clomifene is not effective, metformin may help women with PCOS, especially when linked to insulin resistance.

c. Human menopausal gonadotropin, or hMG (Repronex): This contains both FSH and LH. Patients who do not ovulate because of a fault in the pituitary gland may receive this drug as an injection.

d. Follicle-stimulating hormone (Gonal-F, Bravelle): This hormone is produced by the pituitary gland that controls oestrogen production by the ovaries. It stimulates the ovaries to mature egg follicles.

e. Human chorionic gonadotropin (Ovidrel, Pregnyl): Used together with clomiphene, hMG, and FSH, this can stimulate the follicle to ovulate.

f. Gonadotropin-releasing hormone (Gn-RH) analogs: These can help women who ovulate too early—before the lead follicle is mature—during hmG treatment. It delivers a constant supply of Gn-RH to the pituitary gland, which alters the production of hormone, allowing the doctor to induce follicle growth with FSH.

g. Bromocriptine (Parlodel): This drug inhibits prolactin production. Prolactin stimulates milk production during breastfeeding. Outside pregnancy and lactation, women with high levels of prolactin may have irregular ovulation cycles and fertility problems.

Assisted conception:

The following methods are currently available for assisted conception.

a. Intrauterine insemination (IUI): At the time of ovulation, a fine catheter is inserted through the cervix into the uterus to place a sperm sample directly into the uterus. The sperm is washed in a fluid and the best specimens are selected. The woman may be given a low dose of ovary stimulating hormones. IUI is more commonly done when the man has a low sperm count, decreased sperm motility, or when infertility does not have an identifiable cause. It can also help if a man has severe erectile dysfunction.

b. In-vitro fertilization (IVF): Sperm are placed with unfertilized eggs in a petri dish, where fertilization can take place. The embryo is then placed in the uterus to begin a pregnancy. Sometimes the embryo is frozen for future use.

c. Intracytoplasmic sperm injection (ICSI): A single sperm is injected into an egg to achieve fertilization during an IVF procedure. The likelihood of fertilization improves significantly for men with low sperm concentrations.

d. Sperm or egg donation: If necessary, sperm or eggs can be received from a donor. Fertility treatment with donor eggs is usually done using IVF.

e. Assisted hatching: The embryologist opens a small hole in the outer membrane of the embryo, known as the zona pellucid. The opening improves the ability of the embryo to implant into the uterine lining. This improves the chances that the embryo will implant at, or attach to, the wall of the uterus. This may be used if IVF has not been effective, if there has been poor embryo growth rate, and if the woman is older. In some women, and especially with age, the membrane becomes harder. This can make it difficult for the embryo to implant.

f. Electric or vibratory stimulation to achieve ejaculation: Ejaculation is achieved with electric or vibratory stimulation. This can help a man who cannot ejaculate normally, for example, because of a spinal cord injury.

g. Surgical sperm aspiration: The sperm is removed from part of the male reproductive tract, such as the vas deferens, testicle, or epididymis.

Surgical procedures for women:

If the fallopian tubes are blocked or scarred, surgical repair may make it easier for eggs to pass through. Endometriosis may be treated through laparoscopic surgery. A small incision is made in the abdomen, and a thin, flexible microscope with a light at the end, called a laparoscope, is inserted through it. The surgeon can remove implants and scar tissue, and this may reduce pain and aid fertility.

Probable Questions:

1. Define infertility. What are the types of infertility?
2. Discuss the causes of male sterility.
3. What medicines induce male sterility.
4. Discuss the causes of female sterility.
5. How male sterility can be diagnosed?
6. How female sterility can be diagnosed?
7. What is ectopic pregnancy? Explain.
8. Discuss fertility treatment in females?
9. Discuss fertility treatments in males?

Suggested Readings:

1. Embryology by N. Kumarsen
2. Developmental Biology by Veerbala Rastogi.
3. Embryology by M.P. Arora
4. Developmental Biology by Gilbert.

Unit-II

Assisted Reproductive Technologies : Sperm and ova bank; Artificial Insemination donor (AID); in vitro fertilization (IVF), procedures, variations of IVF, Success rates and complications; Gamete Intrafallopian transfer (GIFT), Intracytoplasmic sperm Injection (ICSI), Surrogate mothers

Objective: In this unit we will discuss bout sperm and ova bank and their utility. We will also discuss about different methods of in vitro fertilization, their types, risk factors and success rate. We will also discuss about surrogacy.

Ova Bank: Egg donation is a process by which women donate eggs for the purposes of assisted reproduction. This process typically involves in vitro fertilisation technology, with the eggs being fertilised in the laboratory. Unfertilised eggs can be frozen and stored for future use at an ova bank. The first child born from egg donation was reported at Monash IVF Clinic, Australia in 1983.

Need for egg donation : This may arise for a number of reasons such as,
a. Infertile couples may resort to egg donation when the female partner cannot have her own genetic children because her own eggs cannot generate a viable pregnancy; or because they could generate a viable pregnancy, but the chances are so low that it is not advisable to do IVF with her own eggs. This situation is often, but not always, based on advanced reproductive age.
b. It can also be due to early onset of menopause, which can occur as early as in women's 20s.
c. In addition, some women are born without ovaries, while some women's reproductive organs have been damaged or surgically removed due to disease or other circumstances.
d. Another indication would be a genetic disorder that either renders her infertile or dangerous for any offspring – problems that can be circumvented by using eggs from another woman.

Sperm Bank:
A sperm bank can freeze and store sperm samples for patients in need of preserving their fertility or for use in fertility treatment. The second purpose is to store donor sperm for use by patients who cannot otherwise conceive children. The sperm bank offers fertility preservation for men who are undergoing either surgery or treatment that may cause permanent changes in their chances of conceiving children, either by affecting the sperm production process, i.e. chemotherapy, orchiectomy or testosterone medication; or where the ducts are cut or clogged, i.e. vasectomy. Short-term storage of semen is also offered to patients who have busy travelling schedules or who live abroad. The sperm can then be used for artificial inseminations, IVF or ICSI even though the husband/male partner is away. In some case where sperm counts are very low, sperm is stored as a back-up in case the count is even lower at the time of ICSI. Back-up storage is also done when a man struggles with passing a sample, then the pressure is off on the day when sperm are needed for ICSI or AI. The second purpose of a sperm bank is the

accumulation of donated sperm. Sperm donation is most probably one of the oldest forms of fertility treatment. In some cases a sperm donor is the only way of conception, e.g. for azoospermic males, i.e. no sperm production is present on confirmation of a testicular biopsy; and in homosexual female couples. In cases of a very low sperm count, ICSI would be the optimal treatment, but it might not be possible for the couple because of financial constraints or religious views. In such situations, artificial insemination with donor sperm can be considered an alternative treatment option.

In Vitro Fertilization (IVF):

In Vitro Fertilization is an assisted reproductive technology (ART) commonly referred to as IVF. IVF is the process of fertilization by extracting eggs, retrieving a sperm sample, and then manually combining an egg and sperm in a laboratory dish. The embryo(s) is then transferred to the uterus. Other forms of ART include gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT).

Why is IVF used? IVF can be used to treat infertility in the following patients:

a. Blocked or damaged fallopian tubes
b. Male factor infertility including decreased sperm count or sperm motility
c. Women with ovulation disorders, premature ovarian failure, uterine fibroids
d. Women who have had their fallopian tubes removed
e. Individuals with a genetic disorder
f. Unexplained infertility

The important techniques employed in assisted reproductive technology are listed below:

i. Intrauterine insemination (IUI).
ii. In vitro fertilization and embryo transfer (IVF and ET).
iii. Gamete intra-fallopian transfer (GIFT).
iv. Zygote intra-fallopian transfer (ZIPT).
v. Intra-vaginal culture (IVC).
vi. Cytoplasmic transfer (CT).
vii. Micromanipulation (Intra-cytoplasmic sperm injection (ICSI), sub-zonal insertion (SUZI).
viii. Cryopreservation.
ix. Assisted hatching (AH).

Among these techniques, the most commonly used procedure is in vitro fertilization and embryo transfer. Important features of different types of ART are briefly described.

Intrauterine Insemination (IUI):

The infertile women (due to endometriosis, idiopathic infertility) without blockage or damage to fallopian tubes can be effectively treated by intrauterine insemination. The women with adequate ovulation and below the age of 40 years are considered for IUI.

The women are usually super-ovulated by administering gonadotrophins. This results in multiple egg development. The IUI is timed to coincide with ovulation. The semen is washed and the highly motile sperms are separated. By using a thin and soft catheter, the sperms are

placed either in the cervix or in uterine cavity. The women subjects are advised to remain lying down for about 15-30 minutes following IUI.
Insemination should be carefully timed for good success. If it is done, a little before the expected time of ovulation, the chances for fertilization are much higher. IUI is usually successful in the first 3-4 attempts. In any case, this approach is not recommended for more than a maximum of 6 ovulation cycles. The success rates of IUI vary considerably and are in the range of 15-30%.

In Vitro Fertilization and Embryo Transfer (IVF and ET):
In vitro fertilization broadly deals with the removal of eggs from a women, fertilizing them in the laboratory, and then transferring the fertilized eggs (zygotes) into the uterus a few days later.

Indications for IVF:
Infertility due to the following causes may be considered for IVF.
i. Failed ovulation induction
ii. Tubal diseases
iii. Cervical hostility
iv. Endometriosis
v. Idiopathic infertility (in men and women).

Ideal Subjects for IVF:
Although it is not always possible to have a choice in the selection of subjects, the following criteria are preferred.

i. Woman below 35 years.
ii. Presence of at least one functional ovary.
iii. Husband with normal motile sperm count.
iv. The couple must be negative for HIV and hepatitis.

Methodology of IVF:
The in vitro fertilization broadly involves the following steps.
1. Induction of superovulation.
2. Monitoring of ovarian response.
3. Oocyte retrieval.
4. Fertilization in vitro.
5. Embryo transfer.

Induction of Superovulation:
It is well known that the success rate IVF is much higher when more embryos (3-5) are transferred. This is possible only with controlled ovarian hyper-stimulation (COH). The other advantages of COH include improvement in the quality of oocyte, control of ovulation timing, besides overcoming the ovulatory dysfunction. The following drug regimens are in use to induce superovulation.

i. Clomiphene citrate (CC).
ii. CC + human menopausal gonadotrophin (hMG).
iii. CC + follicle stimulating hormone (FSH).
iv. Human menopausal gonadotrophin.
v. Follicle stimulating hormone.
vi. Gonadotrophin releasing hormone agonists (GnRHa) + hMG (or FSH).

It is now common to use GnRH agonists to induce ovulation. These compounds act through a process called down regulation of the physiologic hypothalamic- pituitary-ovarian feedback mechanism to effectively suppress spontaneous ovulation.

Monitoring of Ovarian Response:
The follicular growth or ovarian response can be monitored by increase in serum oestradiol level, increase in follicular diameter and thickening of endometrial bed.

Oocyte Retrieval:
The most common method for oocyte retrieval is carried out through vaginal route under ultrasound guidance. This method is simple and less invasive, and can be performed with analgesics only. It is easy to recognize the oocyte as a single cell surrounded by a mass of cumulus cells. The recovered oocytes are maintained in vitro culture for 4-6 hours.

Fertilization in Vitro:
The semen specimens are collected (just prior to oocyte retrieval) via masturbation, processed, and incubated in protein-supplemented media for 3-4 hours prior to fertilization. The incubation results in sperm capacitation.
The retrieved oocytes are also cultured in protein-supplemented media for about 6-8 hours. For the purpose of IVF, 50,000-1, 00,000 capacitated sperms are placed in culture with a single oocyte. The signs of fertilization may be demonstrated 16-20 hours later by the presence of two pronuclei within the developing embryo. There is no need to change the regime for a single failure of IVF. Many a times, success occurs in the subsequent cycles. The two most important criteria for the success of IVF are sperm density and motility.

Embryo Transfer:
Embryo at a stage between pronuclei and blastocyst stage are transferred. Conventionally, 4- 8 cell stage embryos are transferred between 48-60 hours following insemination. The transfer procedure is carried out by use of a catheter.
Not more than three embryos are transferred (per cycle) to minimize multiple pregnancies. However, in the women above the age of 40 years, higher number of embryo may be transferred. (Note: Excess oocytes and embryos are cryopreserved for further use. This will reduce the cost, besides the risk of ovarian hyper stimulation).
Luteal phase support is given by administration of progesterone for about two weeks. By this time, the diagnosis of pregnancy can be assessed by estimating human chorionic gonadotrophin (hCG).

Success Rates of IVF:

Success of IVF varies from programme to programme and within the same programme, the success rate is dependent on the correct diagnosis of the patient, and age. The overall pregnancy rate in IVF is in the range of 25-35% per oocyte retrieval. The take home baby rate is about 15-20% per procedure.

The success rate of IVF is rather low due to the following reasons:

i. Increased risk of abortion
ii. Multiple pregnancy
iii. Ectopic pregnancy
iv. Low birth weight baby
v. Premature delivery.

The success rate of IVF clinics depends on a number of factors including reproductive history, maternal age, the cause of infertility, and lifestyle factors. It is also important to understand that pregnancy rates are not the same as live birth rates.

In the United States, the live birth rate for each IVF cycle started is approximate:

a. 41-43% for women under age 35
b. 33-36% for women ages 35 to 37
c. 23-27% for women ages 38 to 40
d. 13-18% for women ages over 40

The World's Picture of Test Tube Babies:

By employing in vitro fertilization and embryo transfer, the world's first test tube baby (Louise Brown) was born in UK on 28th July 1978. The world's second test tube baby (Kanupriya alias Durga) was born in Kolkata on 3rd October 1978. A team led by Subhash Mukherjee carried IVF and ET in India. Scientists responsible for the "birth of test tube babies were severely criticized then. In fact, IVF turned out to be one of the major achievements of medical sciences in the last century. It has become a novel way of treating infertility. Today, there are more than a million test tube babies born all over the world. In 2003, the world celebrated the silver jubilee of IVF with much fanfare.

Gamete Intra-Fallopian Transfer (GIFT):

Gamete intra-fallopian transfer involves the transfer of both sperm and unfertilized oocyte into the fallopian tube. This allows the fertilization to naturally occur in vivo. The prerequisite for GIFT procedure is that the woman should have at least one normal fallopian tube.

The induction of ovulation and the monitoring procedures for GIFT are almost the same as described for IVF. A couple of hours prior to oocyte retrieval, semen specimens are collected. Two oocytes along with 2-5 lakhs motile sperms for each fallopian tube are placed in a plastic tube container. It is then inserted (by laparoscopy) 4 cm into the distal end of the fallopian tube, and the oocyte sperm combination is injected. The overall pregnancy rate is as high as 30- 40%. The take home baby rate is about 25%. This is much higher when compared to IVF. But the major limitation is the requirement of laparoscopy (a major surgical procedure) to transfer oocytes and sperms into the fallopian tubes.

Zygote Intra-Fallopian Transfer (ZIFT):
ZIFT is suitable when the infertility lies in men, or in case of failure of GIFT.
The wife's oocytes are exposed to her husband's sperms in the laboratory. The fertilized eggs (zygotes) within 24 hours are transferred to the fallopian tube by using laparoscopy.
ZIPT has an advantage over GIFT with male factor infertility. Further, it can be known whether the wife's oocytes have been fertilized by her husbands' sperms.

Intra Vaginal Culture (IVC):
The body's own environment is appropriately utilized in intra-vaginal culture. The retrieved oocytes and sperms are placed in a culture medium inside a sealed container. This is inserted into the vagina. The container is held by a vaginal diaphragm. Thus, the oocytes and sperms are maintained at the normal body temperature (in contrast to any incubator in the laboratory). Two to 3 three days later, the container is opened, and the fertilized and dividing zygotes are transferred into the uterus. This procedure appears simple, but the success rate is very low.

Cytoplasmic Transfer (CT):
Cytoplasm includes many things, the most important being mitochondria which provide energy to the cell. It is possible that deficiency in the mitochondria may leave the oocyte without the necessary power for cell division, after fertilization. This may result in abnormal cell division and poor development of embryo. It is therefore logical to think of the transfer of cytoplasm from a donor (with active mitochondria) into the oocyte of a woman. The advantage with cytoplasmic transfer is that the mother's own genetic material is passed on to the offspring.

Two methods of cytoplasmic transfer have been developed:
1. Transfer of a small amount of cytoplasm by a tiny needle from a donor to a recipient oocyte.

2. Transfer of a large amount of cytoplasm which is fused with the recipient's cytoplasm by applying electricity.

The procedure of cytoplasmic transfer is tedious and technically difficult, besides the cost factor. At least two viable pregnancies have been so far reported in literature by this approach.

Micromanipulation:
Micromanipulation involves in vitro micro-surgically assisted fertilization procedures. This is required when the sperms are unable to penetrate the zona pellucida of oocyte and fertilize. Micromanipulations are usually done in severe cases of male factor infertility. A diagrammatic representation of micromanipulation is depicted in Fig 18.3.

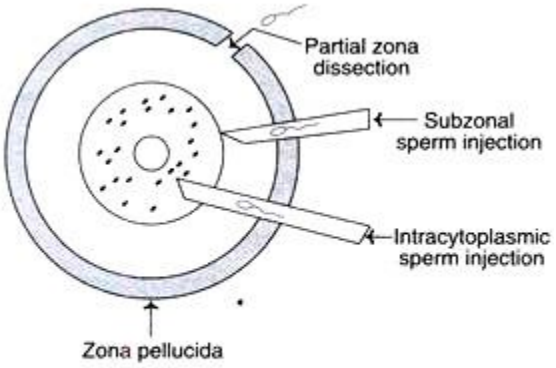


Fig. 18.3 : Micromanipulation for fertilization of an egg (microsurgically assisted fertilization).

Intra-Cytoplasmic Sperm Injection (ICSI):

Intra-cytoplasmic sperm injection is a new and novel infertility treatment utilizing the micromanipulation technology. Many of the previous treatment processes for male infertility have been abandoned in favour of ICSI. The male factor infertility could be due to low sperm counts, poor sperm motility, and poor quality of sperm to penetrate oocyte.

By partial zona dissection (PZD), the zona pellucida is opened using either chemical dissolution or a sharp instrument. A single spermatozoon can be directly injected into the cytoplasm of the oocyte through the micro-puncture of zona pellucida. A micropipette is used to hold the oocyte while the spermatozoon is deposited inside the ooplasm of the oocyte. Besides using normal sperms, round-headed sperms, sperms collected directly from the epididymis and previously cryopreserved sperms can be used in ICSI.

Among the micromanipulation techniques ICSI is the most successful one with a fertilization rate of about 65%. Attempts are on to improve this further. In fact, ICSI has revolutionized assistant reproductive technology by utilizing the sperms of husbands who were once considered to be unsuitable for fertilization process.

Sub-zonal Insertion (SUZI):

In sub-zonal insertion, the zona pellucida is punctured and sperms (1-30 in number) are injected into an area between the zona and the egg. It is expected that one of the sperms will fertilize the egg. The major limitation of SUZI is polyspermy since it is not possible to control the number of sperms that enter the egg.

Round Spermatid nucleus Injection (ROSNI):

There are a few men who cannot manufacture sperms, and therefore they have a zero sperm count. For these men, it is possible to take out the round spermatids (immature cells) directly from the testicle, isolate the nucleus (containing the genetic material) and inject it into the partner's eggs. ROSNI is a recent exciting breakthrough to solve the problem of male infertility through micromanipulation.

Cryopreservation:
Preservation in a frozen state is regarded as cryopreservation. Cryopreservation is very useful in assisted reproductive technology.

i. Semen can be cryopreserved. This may be from the donors, cancer patients (before the commencement of treatment).
ii. Fertilized eggs after IVP or ICSI can be preserved.
iii. Embryos can also be preserved for transfer at a later stage.

Human embryos have been successfully preserved in the presence of cryoprotectants (1, 2-propanediol/dimethyl sulfoxide/glycerol) and stored at -196°C under liquid nitrogen. At appropriate time, the embryos are thawed, cryoprotectants removed and then transferred. Many test tube babies in fact have been born as a result of application of freezing technology.

Assisted Hatching (AH):
Improper implantation of the embryo in the uterus is one of the limiting factors in the success of ART in humans. Assisted hatching is a novel approach for the proper implantation of the embryo in the endometrium.
The embryos in the uterus possess an outer coating namely zona pellucida (the shell). These embryos must be hatched to remove the shell, a step necessary for implantation. In certain women, particularly above 40 years age, natural hatching does not occur, and requires outside assistance. Assisted hatching is carried out by using a Laser to make a small hole in the shell of the embryo. These embryos when transferred into the uterus hatch and get implanted. During the course of AH for 3-4 days, the women are kept on steroids (to suppress mother's immunity) and antibiotics (to counter infections). Better results are reported with this approach.

Pre-implantation Genetic Diagnosis (PGD):
The genetic defects in ovum before fertilization or in the embryo before implantation can be identified by a new medical tool namely pre-implantation genetic diagnosis. It is estimated that about 60% of the ART driven pregnancies are lost due to chromosomal abnormalities. This can be minimized or prevented by using PGD. A direct determination of chromosomal abnormalities prior to implantation ensures a successful pregnancy and ultimate delivery of a healthy baby. One group of workers has reported an increase in the pregnancy rate from 15 to 30% by employing pre-implantation genetic diagnosis.

DNA Amplification and Analysis:
The latest in PGD is the direct DNA analysis. This can be carried out by removing a single cell from 6-8-cell embryo. The DNA is removed and amplified by employing polymerase chain reaction. Direct DNA analysis is useful for the diagnosis of several genetic diseases e.g. cystic fibrosis, sickle-cell anaemia, haemophilia, Duchene's muscular dystrophy, Tay-Sachs disease.

Ethical Advantages of PGD:
PGD is highly advantageous from the ethical point of view, since the embryos with genetic disorders can be discarded in the very stages without the formation of offspring's with undesirable characteristics.

The Negative Aspects of Art:
There are certain limitations/disadvantages associated with assisted reproductive technology in humans. Some highlights are given. It must however, be noted that the advantages of ART outweigh the disadvantages.

Ovarian Hyper-stimulation Syndrome (OHSS):
Due to administration of hormones and drugs, ovarian hyper-stimulation is frequently associated with complications, sometimes even life- threatening. OHSS is more severe in women who conceived in the same cycle, and received hCG as luteal support (following embryo transfer).

Risks Associated with Pregnancy:
ART is associated with multiple pregnancy, increased risk for anaemia, gestational diabetes and premature labour. Low birth weight and prematurity are closed linked with mortality and morbidity.

Premature Menopause:
Controlled ovarian hyper-stimulation (COH) causes multiple follicular utility. There is a risk of premature menopause as COH may reduce the ovarian follicles, besides faster aging. Sometimes, a single COH may use ovarian follicles, which in the normal course are equivalent to two years of ovulation during the natural menstrual cycle.

Ovarian Cancer:
The use of fertility drugs and injuries to epithelium increase the risk of ovarian cancer at least by three times when compared to normal women.

Side effects of in vitro fertilization
Although you may need to take it easy after the procedure, most women can resume normal activities the following day.

Some side effects after IVF may include:

1. Passing a small amount of fluid (may be clear or blood-tinged) after the procedure
2. Mild cramping
3. Mild bloating
4. Constipation
5. Breast tenderness

Some side effects of fertility medications may include:

a. Headaches
b. Mood swings

c. Abdominal pain
d. Hot flashes
e. Abdominal bloating
f. Rare: Ovarian hyperstimulation syndrome (OHSS)

Risks associated with in vitro fertilization:
As with most medical procedures, there are potential risks. More severe symptoms, typically from OHSS, include the following:
a. Nausea or vomiting
b. Decreased urinary frequency
c. Shortness of breath
d. Faintness
e. Severe stomach pains and bloating
f. Ten-pound weight gain within three to five days
If you experience any of these symptoms above, contact your doctor right away.

Additional risks of IVF include the following:
a. Egg retrieval carries risks of bleeding, infection, and damage to the bowel or bladder.
b. The chance of a multiples pregnancy is increased with the use of fertility treatment. There are additional risks and concerns related to multiples during pregnancy including the increased risk of premature delivery and low birth weight.
c. Though the rates of miscarriage are similar to unassisted conception, the risk does increase with maternal age.
d. The Mayo Clinic reports that the risk of ectopic pregnancy with IVF is 2-5%. An ectopic pregnancy is when a fertilized egg implants anywhere outside the uterus and is not viable.
e. Assisted reproductive technology (ART) involves a significant physical, financial, and emotional commitment on the part of a couple. Psychological stress and emotional problems are common, especially if in vitro fertilization (IVF) is unsuccessful.
f. IVF is expensive, and many insurance plans do not provide coverage for fertility treatment. The cost for a single IVF cycle can range from at least $12,000-$17,000.

Surrogacy:

Surrogacy is an arrangement, often supported by a legal agreement, whereby a woman (the surrogate mother) agrees to bear a child for another person or persons, who will become the child's parent(s) after birth. People may seek a surrogacy arrangement when pregnancy is medically impossible, when pregnancy risks are too dangerous for the intended mother, or when a single man or a male couple wish to have a child. Surrogacy is considered one of many assisted reproductive technologies. In surrogacy arrangements, monetary compensation may or may not be involved. Receiving money for the arrangement is known as commercial surrogacy. The legality and cost of surrogacy varies widely between jurisdictions, sometimes resulting in problematic international or interstate surrogacy arrangements. Couples seeking a

surrogacy arrangement in a country where it is banned sometimes travel to a jurisdiction that permits it. In some countries, surrogacy is only legal if money does not exchange hands.

Where commercial surrogacy is legal, couples may use the help of third-party agencies to assist in the process of surrogacy by finding a surrogate and arranging a surrogacy contract with her. These agencies often screen surrogates' psychological and other medical tests to ensure the best chance of healthy gestation and delivery. They also usually facilitate all legal matters concerning the intended parents and the surrogate.

Indications for surrogacy:

Opting for surrogacy is often a choice made when women are unable to carry children on their own. This can be for a number of reasons, including an abnormal uterus or a complete absence of a uterus either congenitally (also known as Mayer-Roakitansky-Kuster-Hauser syndrome) or post-hysterectomy. Women may have a hysterectomy due to complications in childbirth such as heavy bleeding or a ruptured uterus. Medical diseases such as cervical cancer or endometrial cancer can also lead to surgical removal of the uterus. Past implantation failures, history of multiple miscarriages, or concurrent severe heart or renal conditions that can make pregnancy harmful may also prompt women to consider surrogacy. The biological impossibility of single men and same-sex couples having a baby also may indicate surrogacy as an option.

Types of Surrogacy:

There are two types of surrogacy — traditional surrogacy and gestational surrogacy. In traditional surrogacy, a surrogate mother is artificially inseminated, either by the intended father or an anonymous donor, and carries the baby to term. The child is thereby genetically related to both the surrogate mother, who provides the egg, and the intended father or anonymous donor.

In gestational surrogacy, an egg is removed from the intended mother or an anonymous donor and fertilized with the sperm of the intended father or anonymous donor. The fertilized egg, or embryo, is then transferred to a surrogate who carries the baby to term. The child is thereby genetically related to the woman who donated the egg and the intended father or sperm donor, but not the surrogate. Some lesbian couples find gestational surrogacy attractive because it permits one woman to contribute her egg and the other to carry the child. Traditional surrogacy is more controversial than gestational surrogacy, in large part because the biological relationship between the surrogate and the child often complicates the facts of the case if parental rights or the validity of the surrogacy agreement are challenged. As a result, most states prohibit traditional surrogacy agreements. Additionally, many states that permit surrogacy agreements prohibit compensation beyond the payment of medical and legal expenses incurred as a result of the surrogacy agreement.

Qualification of a surrogate mother:

Most surrogacy agencies and fertility clinics require surrogates to meet the following general qualifications:

a. Be in good physical and mental health;
b. Have carried and delivered at least one child;
c. Have had pregnancies that were all free of complications and were full-term;
d. Be less than 43 years of age (some clinics will accept older woman in certain circumstances; others have younger age cut-offs for all surrogates);
e. Be in a stable living situation; and
f. Not smoke or abuse alcohol.

Risks of surrogacy:

The embryo implanted in gestational surrogacy faces the same risks as anyone using IVF would. Preimplantation risks of the embryo include unintentional epigenetic effects, influence of media which the embryo is cultured on, and undesirable consequences of invasive manipulation of the embryo. Often, multiple embryos are transferred to increase the chance of implantation, and if multiple gestations occur, both the surrogate and the embryos face higher risks of complications.

Gestational surrogates have a smaller chance of having hypertensive disorder during pregnancy compared to mothers pregnant by oocyte donation. This is possibly because surrogate mothers tend to be healthier and more fertile than women who use oocyte donation. Surrogate mothers also have low rates of placenta praevia / placental abruptions (1.1-7.9%). Children born through singleton IVF surrogacy have been shown to have no physical or mental abnormalities compared to those children born through natural conception. However, children born through multiple gestation in surrogate mothers often result in preterm labour and delivery, resulting in prematurity and physical and/or mental anomalies.

Probable Questions:

1. What is Ova bank. State its utility.
2. What is sperm bank? State its utility.
3. Define IVF? Why it is needed?
4. Discuss different steps of IVF.
5. What is intrauterine insemination. Discuss its advantages.
6. Why success rate of IVF is low?
7. Discuss Gamete Intra-Fallopian Transfer.
8. Discuss Zygote Intra-Fallopian Transfer.
9. What are the two methods of cytoplasmic transfer?
10. What is Intra-Cytoplasmic Sperm Injection?
11. Discuss Round Spermatid nucleus Injection.
12. Wat is assisted hatching?
13. What are the side effects of In vitro fertilization?
14. Define surrogacy? What are the qualification of a surrogate mother should be ?
15. Discuss the types of surrogacy.
16. Discuss about risks of surrogacy.

Suggested Readings:

1. Biotechnology by P.K. Gupta
2. Gene Cloning by T. Brown.
3. Biotechnology by N. Kumarsen.
4. Biotechnology by B.D. Singh

Unit-III

Genetic errors of human development- Down syndrome, Fragile X syndrome. Techniques used in Medical Embryology : i) Amniocentesis ii) Chorionic villus sampling iii) Ultrasonography.

Objective: In this unit we will discuss about some genetic errors of human development such as Down syndrome, Fragile X syndrome. In addition to that we will discuss about some techniques which are used in medical embryology such as Amniocentesis, Chorionic villus sampling, ultrasonography and DNA finger printing.

A. Down Syndrome:

The first autosomal abnormality described in man by John Langdon Down (1966) was known as Down's syndrome, more commonly used as mongolism. In this case, there is simple trisomy of chromosome 21, i.e. each cell containing three chromosomes of 21 rather than two. The term "Mongolism" has been applied due to their facial characteristics (round, full face with upper eyelids turned downwards), very similar to that of the Mongolian race. It is impossible to be certain whether the trisomy involves chromosome 21 or 22 (of Gr. G) since they are morphologically identical. For this, it is sometimes called G-trisomy.

1. In 1866, Down classified the types of idiots in his "On the ethnological classification of idiots" book.
2. Lejeune (1959) established that this particular class of idiots have three 21 chromosome (chr. or ch.).
3. Yunis (1963, '65) used autoradiography technique at the later part of the "S" period, and labelled 21 chromosome as late replicating.
4. Egasce (1969) said that the size of the 22 is slightly larger than that of 21 chromosome (though controversial). So 21 and 22 differ from each other in their size.
5. Caspersson (1970) used fluorescence microscopy to distinguish 21 chromosome from 22 chromosome. He found that the tip of the long arms of 21 chromosome is more brightly fluorescent than that of 22 chromosome, while short arm is lightly fluorescent in 21 chromosome. So 21 and 22 chromosomes can be distinguished by fluorescent microscope study.
6. Giemsa-banding technique is another technique where chemicals are used for staining and each chromosome pair shows specific banding pattern. Since the fundamental discovery, there has been general agreement that the trisomic chromosome is 21, so that the preferred designation for the syndrome is "trisomy-21".

Physical and Physiological Features of Down's Syndrome:

1. Mentally retarded child.
2. I Q ≤ 50 3)

3. Dull and happy looking.
4. Less sensitive to external stimuli.
5. Individuals having very low birth-weight (mean = 2.83 kg.)
6. Skull is brachycephalic (short from front to back).
7. A round, full face with epicanthic folds in their eyes. Here upper eyelid covers the lower eyelid. Brush filled spots i.e., light speckles around the margin of the iris, are present.
8. Nose from root to tip is short as well as flat and mouth with the lips in the shape of a Cupid's bow.
9. Small, rotated ear.
10. A creased tongue.
11. Short stature, stubby hands and feet.
12. On the palm, the two normal more or less diagonal main creases may be replaced by a single transverse crease — "the so-called Simian crease".
13. Dermatoglyphics increased ulnar loops on fingertips (except the index finger and thumb).
14. Usually loops in the fingertips.
15. atd < may be raised.
16. Little finger short, flexion is usually absent, incurved.
17. Feet normal but with wide gap between the 1st and 2nd toes.
18. A few babies have serious intestinal malformations, such as duodenal atresia.
19. Congenital heart defects.
20. No sexual maturity.
21. In males, testicular degeneration and infertility seem to be the rule, with usually small, undescended testis.
22. In females the labia majora tend to be large and cushion-like and the labia minora are small or absent.

Chromosomal Findings in Down's Syndrome:

There are 4 types of Karyotypes associated with Down's Syndrome. Richards (1967) collected data from 1103 cases. Majority are primary 21 trisomies with no great risk of recurrence, but the other 3 types may all be associated with familial Down's syndrome — in siblings and other relatives if there is an inherited translocation and in the offspring of the patient if he or she is a mosaic.

1. Trisomic — 94.4% (47, XX or XY, G+)
2. t(Dq. Gq) —1.5% 46, XX or XY — D, t (Dq. Gq) +
3. 46(XX or XY) G—, t(Gq. Gq) +1.7%
4. Myxoploid/Mosaic -2.3 to 2.5% (46 XX or XY/47, XX or XY + G)

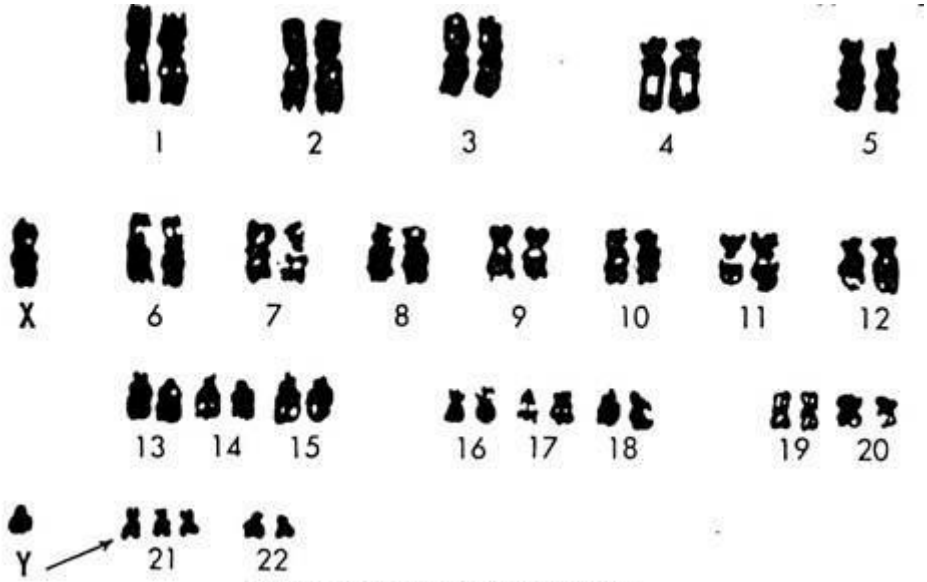


Fig. 9.3 Karyogram of Down's syndrome.

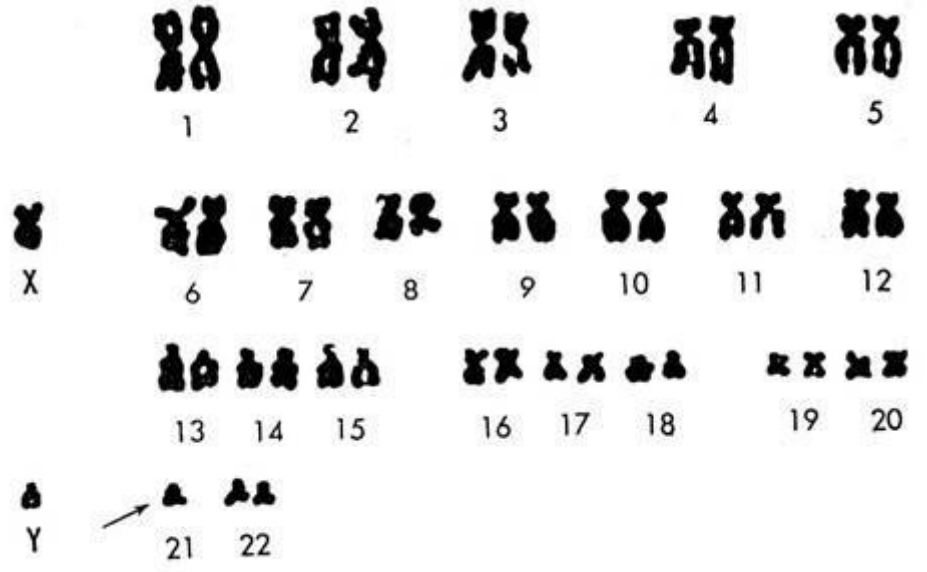


Fig. 9.4 Karyogram of Down's syndrome.

Incidence of Down's Syndrome:

Mongoloid idiocy occurs once in each 500 or 600 births, being more frequent when mothers are older than the average. So, the incidence of mongolism in a mongoloid family depends on the maternal age and not on paternal age.

Age of Mother	Frequency of mongoloid child
20 years	1/30,000
40 years	1/40

Causes of Down's Syndrome:

A. Penrose (1965) has calculated that the risk of chromosomal aberrations which result in Down's syndrome, increase with the maternal age.

He suggests the following possible causes:

i) Ova that become trisomic zygotes are, on the average, shed 10 years later than normal ova.

ii) Due to the presence of a very slowly growing intranuclear virus which divides once in every 2 ½ years, there would be a fourfold increase of risk in every 5 years from the date of first ovulation.

iii) The breakage of the spindle fibres which may be weaken with age. There are some 20 or more strands of spindle fibres attached to each centromere The process of ageing may affect the kinetochore system of at least certain chromosomes.

iv) A specific gene which disturbs the process of cell division may be involved in some cases.

v) Environmental influences such as radiation environmental stress, androgenic hormone increase high fluoride content of water and atmospheric pollution

Some of the developmental and immunological defects associated with Down's Syndrome are hypersensitive to the CMI (Cell multiplication inhibitory) activities of human interferons. B. Hanhart (1961) studied the chromosomes in a mongoloid mother and her affected daughters and demonstrated the presence of secondary nondisjunction.

Diagnosis of Down syndrome:

a. Before birth

When screening tests predict a high risk of Down syndrome, a more invasive diagnostic test (amniocentesis or chorionic villus sampling) is needed to confirm the diagnosis. The false-positive rate with screening is about 2–5% (see section Screening below). Amniocentesis and chorionic villus sampling are more reliable tests, but they increase the risk of miscarriage between 0.5 and 1%. The risk of limb problems may be increased in the offspring if chorionic villus sampling is performed before 10 weeks. The risk from the procedure is greater the earlier it is performed, thus amniocentesis is not recommended before 15 weeks gestational age and chorionic villus sampling before 10 weeks gestational age.

b. After birth

The diagnosis can often be suspected based on the child's physical appearance at birth. An analysis of the child's chromosomes is needed to confirm the diagnosis, and to determine if a translocation is present, as this may help determine the risk of the child's parents having further children with Down syndrome. Parents generally wish to know the possible diagnosis once it is suspected and do not wish pity.

i. Screening

Guidelines recommend screening for Down syndrome to be offered to all pregnant women, regardless of age. A number of tests are used, with varying levels of accuracy. They are typically used in combination to increase the detection rate. None can be definitive, thus if screening is positive, either amniocentesis or chorionic villus sampling is required to confirm

the diagnosis. Screening in both the first and second trimesters is better than just screening in the first trimester. The different screening techniques in use are able to pick up 90–95% of cases, with a false-positive rate of 2–5%. If Down syndrome occurs in one in 500 pregnancies and the test used has a 5% false-positive rate, this means, of 26 women who test positive on screening, only one will have Down syndrome confirmed. If the screening test has a 2% false-positive rate, this means one of eleven who test positive on screening have a foetus with DS

ii. Ultrasound

Ultrasound imaging can be used to screen for Down syndrome. Findings that indicate increased risk when seen at 14 to 24 weeks of gestation include a small or no nasal bone, large ventricles, nuchal fold thickness, and an abnormal right subclavian artery, among others. The presence or absence of many markers is more accurate. Increased foetal nuchal translucency (NT) indicates an increased risk of Down syndrome picking up 75–80% of cases and being falsely positive in 6%.

iii. Blood tests

Several blood markers can be measured to predict the risk of Down syndrome during the first or second trimester. Testing in both trimesters is sometimes recommended and test results are often combined with ultrasound results. In the second trimester, often two or three tests are used in combination with two or three of: α-fetoprotein, unconjugated estriol, total hCG, and free βhCG detecting about 60–70% of cases.

Testing of the mother's blood for fetal DNA is being studied and appears promising in the first trimester. The International Society for Prenatal Diagnosis considers it a reasonable screening option for those women whose pregnancies are at a high risk for trisomy 21. Accuracy has been reported at 98.6% in the first trimester of pregnancy. Confirmatory testing by invasive techniques (amniocentesis, CVS) is still required to confirm the screening result.

Epidemiology:

Down syndrome is the most common chromosomal abnormality in humans. Globally, as of 2010, Down syndrome occurs in about 1 per 1,000 births and results in about 17,000 deaths. More children are born with Down syndrome in countries where abortion is not allowed and in countries where pregnancy more commonly occurs at a later age. About 1.4 per 1,000 live births in the United States and 1.1 per 1,000 live births in Norway are affected. In the 1950s, in the United States, it occurred in 2 per 1000 live births with the decrease since then due to prenatal screening and abortions. The number of pregnancies with Down syndrome is more than two times greater with many spontaneously aborting.[9] It is the cause of 8% of all congenital disorders.

Maternal age affects the chances of having a pregnancy with Down syndrome. At age 20, the chance is 1 in 1,441; at age 30, it is 1 in 959; at age 40, it is 1 in 84; and at age 50 it is 1 in 44. Although the probability increases with maternal age, 70% of children with Down syndrome are born to women 35 years of age and younger, because younger people have more

children. The father's older age is also a risk factor in women older than 35, but not in women younger than 35, and may partly explain the increase in risk as women age

Prognosis:

Between 5 and 15% of children with Down syndrome in Sweden attend regular school. Some graduate from high school; however, most do not. Of those with intellectual disability in the United States who attended high school about 40% graduated. Many learn to read and write and some are able to do paid work. In adulthood about 20% in the United States do paid work in some capacity. In Sweden, however, less than 1% have regular jobs. Many are able to live semi-independently, but they often require help with financial, medical, and legal matters. Those with mosaic Down syndrome usually have better outcomes.

Individuals with Down syndrome have a higher risk of early death than the general population. This is most often from heart problems or infections. Following improved medical care, particularly for heart and gastrointestinal problems, the life expectancy has increased. This increase has been from 12 years in 1912, to 25 years in the 1980s, to 50 to 60 years in the developed world in the 2000s. Currently between 4 and 12% die in the first year of life. The probability of long-term survival is partly determined by the presence of heart problems. In those with congenital heart problems, 60% survive to 10 years and 50% survive to 30 years of age. In those without heart problems, 85% survive to 10 years and 80% survive to 30 years of age. About 10% live to 70 years of age. The National Down Syndrome Society provide information regarding raising a child with Down syndrome

B. Fragile X syndrome:

Fragile X syndrome is one of the most common forms of inherited mental retardation with the estimated incidence of 1 in 4000 males and 1 in 8000 females. The syndrome is transmitted as an X-linked dominant trait and with reduced penetrance (80% in males and 30% in females). Fragile X syndrome is associated with a fragile site, designated FRAXA (Fragile site, X chromosome, A site), at Xq27.3 near the end of the long arm. The clinical presentations of fragile X syndrome include mild to severe mental retardation, with IQ between 20 and 60, mildly abnormal facial features of a prominent jaw and large ears, mainly in males, and macroorchidism in post-pubescent males. Many patients also display subtle connective tissue abnormalities, hyperactive and attention deficit disorder and autistic-like behaviour. In 1991, the molecular basis of fragile X syndrome was revealed by positioning cloning and shown to be associated with a massive trinucleotide repeat expansion within the gene *fragile X mental retardation-1* (*FMR1*). This was one of the first identified human disorders caused by dynamic mutation, trinucleotide repeat expansion.

Fragile X syndrome has an X-linked dominant inheritance. It is typically caused by an expansion of the CGG triplet repeat within the *FMR1* (fragile X mental retardation 1) gene on the X chromosome. This results in silencing (methylation) of this part of the gene and a deficiency of the resultant protein (FMRP), which is required for the normal development

of connections between neurons. Diagnosis requires genetic testing to determine the number of CGG repeats in the *FMR1* gene. Normally, there are between 5 and 40 repeats; fragile X syndrome occurs with more than 200. A premutation is said to be present when the gene has between 40 and 200 repeats; women with a premutation have an increased risk of having an affected child. Testing for premutation carriers may allow for genetic counselling.

There is no cure. Early intervention is recommended, as it provides the most opportunity for developing a full range of skills. These interventions may include special education, speech therapy, physical therapy, or behavioural therapy. Medications may be used to treat associated seizures, mood problems, aggressive behaviour, or ADHD. Fragile X syndrome is estimated to occur in 1.4 in 10,000 males and 0.9 in 10,000 females.

History:

In 1943, British neurologist James Purdon Martin and British geneticist Julia Bell described a pedigree of X-linked mental disability, without considering the macroorchidism (larger testicles). In 1969, Herbert Lubs first sighted an unusual "marker X chromosome" in association with mental disability. In 1970, Frederick Hecht coined the term "fragile site". And, in 1985, Felix F. de la Cruz outlined extensively the physical, psychological, and cytogenic characteristics of those afflicted in addition to prospects for therapy. Continued advocacy later won him an honour through the FRAXA Research Foundation in December 1998

Signs and Symptoms:

Most young children do not show any physical signs of FXS. It is not until puberty that physical features of FXS begin to develop. Aside from intellectual disability, prominent characteristics of the syndrome may include an elongated face, large or protruding ears, flat feet, larger testes (macroorchidism), and low muscle tone. Recurrent otitis media (middle ear infection) and sinusitis is common during early childhood. Speech may be cluttered or nervous. Behavioural characteristics may include stereotypic movements (e.g., hand-flapping) and atypical social development, particularly shyness, limited eye contact, memory problems, and difficulty with face encoding. Some individuals with fragile X syndrome also meet the diagnostic criteria for autism.

Males with a full mutation display virtually complete penetrance and will therefore almost always display symptoms of FXS, while females with a full mutation generally display a penetrance of about 50% as a result of having a second, normal X chromosome. Females with FXS may have symptoms ranging from mild to severe, although they are generally less affected than males.

Physical phenotype:

- Large, protruding ears (both)
- Long face (vertical maxillary excess)
- High-arched palate (related to the above)
- Hyperextensible finger joints

- Hyperextensible ('double-jointed') thumbs
- Flat feet
- Soft skin
- Postpubescent macroorchidism (large testicles in men after puberty)[14]
- Hypotonia (low muscle tone).

Intellectual development:

Individuals with FXS may present anywhere on a continuum from learning disabilities in the context of a normal intelligence quotient (IQ) to severe intellectual disability, with an average IQ of 40 in males who have complete silencing of the *FMR1* gene. Females, who tend to be less affected, generally have an IQ which is normal or borderline with learning difficulties. The main difficulties in individuals with FXS are with working and short-term memory, executive function, visual memory, visual-spatial relationships, and mathematics, with verbal abilities being relatively spared.

Data on intellectual development in FXS are limited. However, there is some evidence that standardized IQ decreases over time in the majority of cases, apparently as a result of slowed intellectual development. A longitudinal study looking at pairs of siblings where one child was affected and the other was not found that affected children had an intellectual learning rate which was 55% slower than unaffected children. When both autism and FXS are present, a greater language deficit and lower IQ is observed as compared to children with only FXS. Individuals with FXS often demonstrated language and communicative problems. This may be related to muscle function of the mouth and frontal-lobe deficits.

Social interaction:

FXS is characterized by social anxiety, including poor eye contact, gaze aversion, prolonged time to commence social interaction, and challenges forming peer relationships. Social anxiety is one of the most common features associated with FXS, with up to 75% of males in one series characterized as having excessive shyness and 50% having panic attacks. Social anxiety in individuals with FXS is related to challenges with face encoding, the ability to recognize a face that one has seen before. It appears that individuals with FXS are interested in social interaction and display greater empathy than groups with other causes of intellectual disability, but display anxiety and withdrawal when placed in unfamiliar situations with unfamiliar people. This may range from mild social withdrawal, which is predominantly associated with shyness, to severe social withdrawal, which may be associated with co-existing autism spectrum disorder.

Females with FXS frequently display shyness, social anxiety and social avoidance or withdrawal. In addition, premutation in females has been found to be associated with social anxiety. Individuals with FXS show decreased activation in the prefrontal regions of the brain.

Mental health:

Attention deficit hyperactivity disorder (ADHD) is found in the majority of males with FXS and 30% of females, making it the most common psychiatric diagnosis in those with FXS. Children with fragile X have very short attention spans, are hyperactive, and show hypersensitivity to visual, auditory, tactile, and olfactory stimuli. These children have difficulty in large crowds due to the loud noises and this can lead to tantrums due to hyperarousal. Hyperactivity and disruptive behaviour peak in the preschool years and then gradually decline with age, although inattentive symptoms are generally lifelong.

Aside from the characteristic social phobia features, a range of other anxiety symptoms are very commonly associated with FXS, with symptoms typically spanning a number of psychiatric diagnoses but not fulfilling any of the criteria in full. Children with FXS pull away from light touch and can find textures of materials to be irritating. Transitions from one location to another can be difficult for children with FXS. Behavioural therapy can be used to decrease the child's sensitivity in some cases. Behaviours such as hand flapping and biting, as well as aggression, can be an expression of anxiety.

Perseveration is a common communicative and behavioural characteristic in FXS. Children with FXS may repeat a certain ordinary activity over and over. In speech, the trend is not only in repeating the same phrase but also talking about the same subject continually. Cluttered speech and self-talk are commonly seen. Self-talk includes talking with oneself using different tones and pitches.[15] Although only a minority of FXS cases will meet the criteria for obsessive–compulsive disorder (OCD), a significant majority will have symptoms of obsession. However, as individuals with FXS generally find these behaviours pleasurable, unlike individuals with OCD, they are more frequently referred to as stereotypic behaviours.

Mood symptoms in individuals with FXS rarely meet diagnostic criteria for a major mood disorder as they are typically not of sustained duration. Instead, these are usually transient and related to stressors, and may involve labile (fluctuating) mood, irritability, self-injury and aggression. Individuals with fragile X-associated tremor/ataxia syndrome (FXTAS) are likely to experience combinations of dementia, mood, and anxiety disorders. Males with the *FMR1* premutation and clinical evidence of FXTAS were found to have increased occurrence of somatization, obsessive–compulsive disorder, interpersonal sensitivity, depression, phobic anxiety, and psychoticism.

Vision:

Ophthalmologic problems include strabismus. This requires early identification to avoid amblyopia. Surgery or patching are usually necessary to treat strabismus if diagnosed early. Refractive errors in patients with FXS are also common.

Neurology:

Individuals with FXS are at a higher risk of developing seizures, with rates between 10% and 40% reported in the literature.[28] In larger study populations the frequency varies between 13% and 18%,[12][28] consistent with a recent survey of caregivers which found that 14% of males and 6% of females experienced seizures.[28] The seizures tend to be partial, are generally not frequent, and are amenable to treatment with medication.

Individuals who are carriers of premutation alleles are at risk for developing fragile X-associated tremor/ataxia syndrome (FXTAS), a progressive neurodegenerative disease. It is seen in approximately half of male carriers over the age of 70, while penetrance in females is lower. Typically, onset of tremor occurs in the sixth decade of life, with subsequent progression to ataxia (loss of coordination) and gradual cognitive decline.

Working memory:

From their 40s onward, males with FXS begin developing progressively more severe problems in performing tasks that require the central executive of working memory. Working memory involves the temporary storage of information 'in mind', while processing the same or other information. Phonological memory (or verbal working memory) deteriorates with age in males, while visual-spatial memory is not found to be directly related to age. Males often experience an impairment in the functioning of the phonological loop. The CGG length is significantly correlated with central executive and the visual–spatial memory. However, in a premutation individual, CGG length is only significantly correlated with the central executive, not with either phonological memory or visual–spatial memory.

Fertility:

About 20% of women who are carriers for the fragile X premutation are affected by fragile X-related primary ovarian insufficiency (FXPOI), which is defined as menopause before the age of 40. The number of CGG repeats correlates with penetrance and age of onset. However premature menopause is more common in premutation carriers than in women with the full mutation, and for premutations with more than 100 repeats the risk of FXPOI begins to decrease. Fragile X-associated primary ovarian insufficiency (FXPOI) is one of three Fragile X-associated Disorders (FXD) caused by changes in the FMR1 gene. FXPOI affects female premutation carriers of Fragile X syndrome, which is caused by the FMR1 gene, when their ovaries are not functioning properly. Women with FXPOI may develop menopause-like symptoms but they are not actually menopausal. Women with FXPOI can still get pregnant in some cases because their ovaries occasionally release viable eggs. FMRP is a chromatin-binding protein that functions in the DNA damage response. FMRP also occupies sites on meiotic chromosomes and regulates the dynamics of the DNA damage response machinery during spermatogenesis

Cause of Fragile X Syndrome:

Fragile X syndrome is a genetic disorder which occurs as a result of a mutation of the *fragile X mental retardation 1* (*FMR1*) gene on the X chromosome, most commonly an increase in the number of CGG trinucleotide repeats in the 5' untranslated region of *FMR1*. Mutation at that site is found in 1 out of about every 2000 males and 1 out of about every 259 females. Incidence of the disorder itself is about 1 in every 3600 males and 1 in 4000–6000 females. Although this accounts for over 98% of cases, FXS can also occur as a result of point mutations affecting *FMR1*.

In unaffected individuals, the *FMR1* gene contains 5–44 repeats of the sequence CGG, most commonly 29 or 30 repeats. Between 45-54 repeats is considered a "grey zone", with a premutation allele generally considered to be between 55 and 200 repeats in length. Individuals with fragile X syndrome have a full mutation of the *FMR1* allele, with over 200 CGG repeats. In these individuals with a repeat expansion greater than 200, there is methylation of the CGG repeat expansion and *FMR1* promoter, leading to the silencing of the *FMR1* gene and a lack of its product.

This methylation of *FMR1* in chromosome band Xq27.3 is believed to result in constriction of the X chromosome which appears 'fragile' under the microscope at that point, a phenomenon that gave the syndrome its name. One study found that FMR1 silencing is mediated by the FMR1 mRNA. The FMR1 mRNA contains the transcribed CGG-repeat tract as part of the 5' untranslated region, which hybridizes to the complementary CGG-repeat portion of the FMR1 gene to form an RNA·DNA duplex. A subset of people with intellectual disability and symptoms resembling fragile X syndrome are found to have point mutations in *FMR1*. This subset lacked the CGG repeat expansion in *FMR1* traditionally associated with fragile x syndrome.[

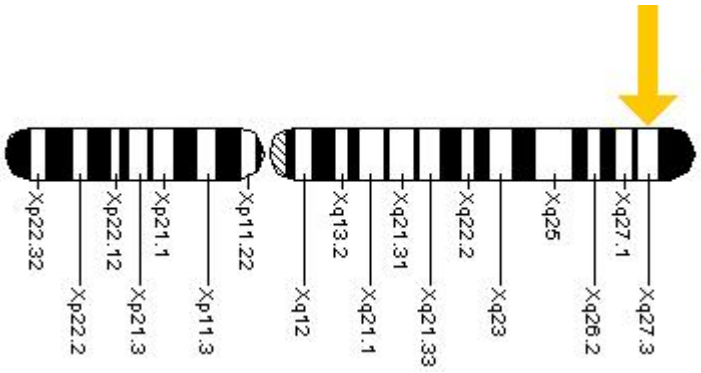


Figure: Location of the FMR1 gene on the X chromosome

Inheritance:

Fragile X syndrome has traditionally been considered an X-linked dominant condition with variable expressivity and possibly reduced penetrance. However, due to genetic anticipation and X-inactivation in females, the inheritance of Fragile X syndrome does not follow the usual pattern of X-linked dominant inheritance, and some scholars have suggested discontinuing labelling X-linked disorders as dominant or recessive. Females with full FMR1 mutations may have a milder phenotype than males due to variability in X-inactivation.

Before the *FMR1* gene was discovered, analysis of pedigrees showed the presence of male carriers who were asymptomatic, with their grandchildren affected by the condition at a higher rate than their siblings suggesting that genetic anticipation was occurring. This tendency for future generations to be affected at a higher frequency became known as the Sherman paradox after its description in 1985. Due to this, male children often have a greater degree of symptoms than their mothers.

The explanation for this phenomenon is that male carriers pass on their premutation to all of their daughters, with the length of the *FMR1* CGG repeat typically not increasing during meiosis, the cell division that is required to produce sperm. Incidentally, males with a full mutation only pass on pre-mutations to their daughters. However, females with a full mutation are able to pass this full mutation on, so theoretically there is a 50% chance that a child will be affected. In addition, the length of the CGG repeat frequently does increase during meiosis in female premutation carriers due to instability and so, depending on the length of their premutation, they may pass on a full mutation to their children who will then be affected. Repeat expansion is considered to be a consequence of strand slippage either during DNA replication or DNA repair synthesis.

Diagnosis:

Cytogenetic analysis for fragile X syndrome was first available in the late 1970s when diagnosis of the syndrome and carrier status could be determined by culturing cells in a folate deficient medium and then assessing for "fragile sites" (discontinuity of staining in the region of the trinucleotide repeat) on the long arm of the X chromosome. This technique proved unreliable, however, as the fragile site was often seen in less than 40% of an individual's cells. This was not as much of a problem in males, but in female carriers, where the fragile site could generally only be seen in 10% of cells, the mutation often could not be visualised.

Since the 1990s, more sensitive molecular techniques have been used to determine carrier status. The fragile X abnormality is now directly determined by analysis of the number of CGG repeats using polymerase chain reaction (PCR) and methylation status using Southern blot analysis. By determining the number of CGG repeats on the X chromosome, this method allows for more accurate assessment of risk for premutation carriers in terms of their own risk of fragile X associated syndromes, as well as their risk of having affected children. Because this method only tests for expansion of the CGG repeat, individuals with FXS due to missense mutations or deletions involving *FMR1* will not be diagnosed using this test and should therefore undergo sequencing of the FMR1 gene if there is clinical suspicion of FXS.

Prenatal testing with chorionic villus sampling or amniocentesis allows diagnosis of FMR1 mutation while the foetus is in utero and appears to be reliable. Early diagnosis of fragile X syndrome or carrier status is important for providing early intervention in children or foetuses with the syndrome, and allowing genetic counselling with regards to the potential for a couple's future children to be affected. Most parents notice delays in speech and language skills, difficulties in social and emotional domains as well as sensitivity levels in certain situations with their children.

Pathophysiology:

FMRP is found throughout the body, but in highest concentrations within the brain and testes. It appears to be primarily responsible for selectively binding to around 4% of mRNA in mammalian brains and transporting it out of the cell nucleus and to the synapses of neurons. Most of these mRNA targets have been found to be located in the dendrites of neurons, and brain tissue from humans with FXS and mouse models shows abnormal dendritic spines, which

are required to increase contact with other neurons. The subsequent abnormalities in the formation and function of synapses and development of neural circuits result in impaired neuroplasticity, an integral part of memory and learning. Connectome changes have long been suspected to be involved in the sensory pathophysiology and most recently a range of circuit alterations have been shown, involving structurally increased local connectivity and functionally decreased long-range connectivity.

In addition, FMRP has been implicated in several signalling pathways that are being targeted by a number of drugs undergoing clinical trials. The group 1 metabotropic glutamate receptor (mGluR) pathway, which includes mGluR1 and mGluR5, is involved in mGluR-dependent long term depression (LTD) and long term potentiation (LTP), both of which are important mechanisms in learning.[11][13] The lack of FMRP, which represses mRNA production and thereby protein synthesis, leads to exaggerated LTD. FMRP also appears to affect dopamine pathways in the prefrontal cortex which is believed to result in the attention deficit, hyperactivity and impulse control problems associated with FXS. The downregulation of GABA pathways, which serve an inhibitory function and are involved in learning and memory, may be a factor in the anxiety symptoms which are commonly seen in FXS.

Prognosis:

A 2013 review stated that life expectancy for FXS was 12 years lower than the general population and that the causes of death were similar to those found for the general population.

Techniques used in Medical Embryology:

A. Amniocentesis:

Definition:

Amniocentesis is a procedure used to diagnose foetal defects in the early second trimester of pregnancy. A sample of the amniotic fluid, which surrounds a foetus in the womb, is collected through a pregnant woman's abdomen using a needle and syringe. Tests performed on foetal cells found in the sample can reveal the presence of many types of genetic disorders, thus allowing doctors and prospective parents to make important decisions about early treatment and intervention.

Purpose:

Since the mid-1970s, amniocentesis has been used routinely to test for Down syndrome , by far the most common, nonhereditary, genetic birth defect, afflicting about one in every 1,000 babies. By 1997, approximately 800 different diagnostic tests were available, most of them for hereditary genetic disorders such as Tay-Sachs disease, sickle cell anaemia, haemophilia, muscular dystrophy , and cystic fibrosis **.**

Amniocentesis, often called amnio, is recommended for women who will be older than 35 on their due-date. It is also recommended for women who have already borne children with birth

defects, or when either of the parents has a family history of a birth defect for which a diagnostic test is available. Another reason for the procedure is to confirm indications of Down syndrome and certain other defects which may have shown up previously during routine maternal blood screening. The risk of bearing a child with a nonhereditary genetic defect such as Down syndrome is directly related to a woman's age—the older the woman, the greater the risk. Thirty-five is the recommended age to begin amnio testing because that is the age at which the risk of carrying a foetus with such a defect roughly equals the risk of miscarriage caused by the procedure–about one in 200. At age 25, the risk of giving birth to a child with this type of defect is about one in 1,400; by age 45 it increases to about one in 20. Nearly half of all pregnant women over 35 in the United States undergo amniocentesis and many younger women also decide to have the procedure. Notably, some 75% of all Down syndrome infants born in the United States each year are to women younger than 35.

One of the most common reasons for performing amniocentesis is an abnormal alpha-fetoprotein (AFP) test. Alpha-fetoprotein is a protein produced by the foetus and present in the mother's blood. A simple blood screening, usually conducted around the 15th week of pregnancy, can determine the AFP levels in the mother's blood. Levels that are too high or too low may signal possible foetal defects. Because this test has a high false-positive rate, another test such as amnio is recommended whenever the AFP levels fall outside the normal range. Amniocentesis is generally performed during the 16th week of pregnancy, with results usually available within three weeks. It is possible to perform an amnio as early as the 11th week, but this is not usually recommended because there appears to be an increased risk of miscarriage when done at this time. The advantage of early amnio and speedy results lies in the extra time for decision making if a problem is detected. Potential treatment of the foetus can begin earlier. Important, also, is the fact that elective abortions are safer and less controversial the earlier they are performed.

Precautions:
As an invasive surgical procedure, amnio poses a real, although small, risk to the health of a foetus. Parents must weigh the potential value of the knowledge gained, or indeed the reassurance that all is well, against the small risk of damaging what is in all probability a normal foetus. The serious emotional and ethical dilemmas that adverse test results can bring must also be considered. The decision to undergo amnio is always a matter of personal choice.

Description:

The word amniocentesis literally means "puncture of the amnion," the thin-walled sac of fluid in which a developing foetus is suspended during pregnancy. During the sampling procedure, the obstetrician inserts a very fine needle through the woman's abdomen into the uterus and amniotic sac and withdraws approximately one ounce of amniotic fluid for testing. The relatively painless procedure is performed on an outpatient basis, sometimes using local anaesthesia.

The physician uses ultrasound images to guide needle placement and collect the sample, thereby minimizing the risk of foetal injury and the need for repeated needle insertions. Once the sample is collected, the woman can return home after a brief observation period. She may

be instructed to rest for the first 24 hours and to avoid heavy lifting for two days. The sample of amniotic fluid is sent to a laboratory where foetal cells contained in the fluid are isolated and grown in order to provide enough genetic material for testing. This takes about seven to 14 days. The material is then extracted and treated so that visual examination for defects can be made. For some disorders, like Tay-Sachs, the simple presence of a telltale chemical compound in the amniotic fluid is enough to confirm a diagnosis. Depending on the specific tests ordered, and the skill of the lab conducting them, all the results are available between one and four weeks after the sample is taken. Cost of the procedure depends on the doctor, the lab, and the tests ordered. Most insurers provide coverage for women over 35, as a follow-up to positive maternal blood screening results, and when genetic disorders run in the family. An alternative to amnio, now in general use, is chorionic villus sampling, or CVS, which can be performed as early as the eighth week of pregnancy. While this allows for the possibility of a first trimester abortion, if warranted, CVS is apparently also riskier and is more expensive. The most promising area of new research in prenatal testing involves expanding the scope and accuracy of maternal blood screening as this poses no risk to the foetus.

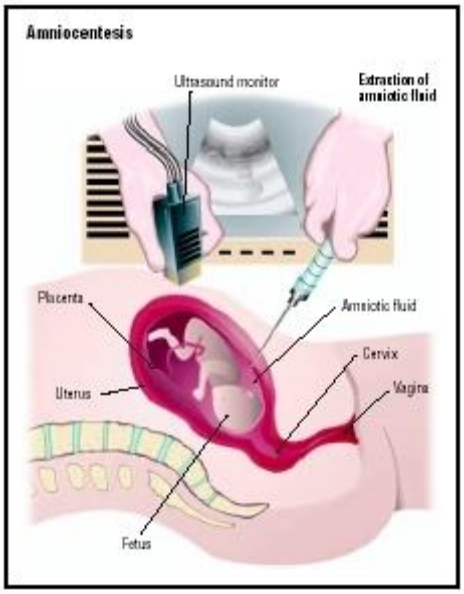


Figure: To perform amniocentesis, a physician uses an ultrasound monitor to visualize the foetus while inserting a syringe to extract amniotic fluid for analysis.

Preparation:

It is important for a woman to fully understand the procedure and to feel confident in the obstetrician performing it. Evidence suggests that a physician's experience with the procedure reduces the chance of mishap. Almost all obstetricians are experienced in performing amniocentesis. The patient should feel free to ask questions and seek emotional support before, during and after the amnio is performed.

Aftercare: Necessary aftercare falls into two categories, physical and emotional.

Physical aftercare: During and immediately following the sampling procedure, a woman may experience dizziness, nausea , a rapid heartbeat, and cramping. Once past these immediate

hurdles, the physician will send the woman home with instructions to rest and to report any complications requiring immediate treatment, including.

- **Vaginal bleeding**: The appearance of blood could signal a problem.
- **Premature labour**: Unusual abdominal pain and/or cramping may indicate the onset of premature labour. Mild cramping for the first day or two following the procedure is normal.
- **Signs of infection**: Leaking of amniotic fluid or unusual vaginal discharge, and fever could signal the onset of infection.

Emotional aftercare: Once the procedure has been safely completed, the anxiety of waiting for the test results can prove to be the worst part of the process. A woman should seek and receive emotional support from family and friends, as well as from her obstetrician and family doctor. Professional counselling may also prove necessary, particularly if a foetal defect is discovered.

Risks of amniocentesis:

Most of the risks and short-term side effects associated with amniocentesis relate to the sampling procedure and have been discussed above. A successful amnio sampling results in no long-term side effects. Risks include:

a. Maternal/foetal haemorrhaging: While spotting in pregnancy is fairly common, bleeding following amnio should always be investigated.
b. Infection: Infection: although rare, can occur after amniocentesis. An unchecked infection can lead to severe complications.
c. Foetal injury: A very slight risk of injury to the fetus resulting from contact with the amnio needle does exist.
d. Miscarriage: The rate of miscarriage occurring during standard, second trimester amnio appears to be approximately 0.5%. This compares to a miscarriage rate of 1% for CVS. Many foetuses with severe genetic defects miscarry naturally during the first trimester.
e. The trauma of difficult family-planning decisions: The threat posed to parental and family mental health from the trauma accompanying an abnormal test result can not be underestimated.

B. Chorionic villus sampling:

Definition:

Chorionic villus sampling (CVS) is a form of prenatal diagnosis to determine genetic abnormalities in the fetus. It entails getting a sample of the chorionic villus (placental tissue) and testing it. It is generally carried out only on pregnant women over the age of 35 and those who have a higher risk of down syndrome and other chromosomal conditions. Chorionic villus sampling (CVS) is a prenatal test that is used to detect birth defects, genetic diseases, and other problems during pregnancy. During the test, a small sample of cells (called chorionic villi) is

taken from the placenta where it attaches to the wall of the uterus. Chorionic villi are tiny parts of the placenta that are formed from the fertilized egg, so they have the same genes as the baby.

The advantage of CVS is that it can be carried out at 10-12 weeks of pregnancy, earlier than amniocentesis (which is carried out at 15-18 weeks). However, it is more risky than amniocentesis, with a 1 in 100 to 200 risk that it will cause a miscarriage.

What Diseases or Disorders Can CVS Identify?

CVS can help identify such chromosomal problems as Down syndrome or other genetic diseases such as cystic fibrosis, Tay-Sachs disease, and sickle cell anemia. CVS is considered to be 98% accurate in the diagnosis of chromosomal defects. The procedure also identifies the sex of the fetus, so it can identify disorders that are linked to one sex (such as certain types of muscular dystrophy that occur most often in males). CVS does not detect open neural tube defects like spina bifida.

Benefits of CVS:

CVS can be done early in pregnancy (earlier than amniocentesis), and results are usually obtained within 10 days. Getting this kind of information early allows a woman to make choices in the beginning stage of her pregnancy. If a woman chooses to terminate the pregnancy after receiving abnormal test results, the termination will be safer than if she waits until later for amniocentesis results.

What kind of problems does CVS diagnose?

Like amniocentesis, CVS can identify:

Nearly all chromosomal abnormalities, including Down syndrome, trisomy 13, trisomy 18, and sex chromosome abnormalities (such as Turner syndrome). The test can diagnose these conditions, but it can't measure their severity.
Several hundred genetic disorders, such as cystic fibrosis, sickle cell disease, and Tay-Sachs disease. The test is not used to look for all of them, but if your baby is at increased risk for one or more of these disorders, CVS can usually tell you whether he has the disease.
Unlike amniocentesis, CVS cannot detect neural tube defects, such as spina bifida. If you opt for CVS, you'll be offered a blood screening test in your second trimester to determine whether you're at increased risk for neural tube defects. Most neural tube defects can be detected by a detailed second-trimester ultrasound done at a state-of-the-art academic center.
Be aware that if you have CVS, there's a 1 to 2 percent chance of getting an unclear result. This is called a confined placental mosaicism, in which some of the cell lines cultured from the placenta contain abnormal chromosomes and some are normal. If your CVS detects a mosaicism, you'll have to have amniocentesis and possibly other testing to determine whether your baby is affected.

Risks of CVS:

There's no consensus on the risk of miscarriage due to CVS, although it's often estimated to be between one in 100 and one in 200 — which is higher than the usual estimates for amniocentesis. But recent research suggests that the risk of miscarriage from CVS is really much lower. One center that does a lot of CVS found the miscarriage rate from the procedure was down to about 1 in 360 — similar to the center's miscarriage rate from amniocentesis. This is most likely due to improvements in ultrasound imaging and the doctors' increased experience in doing CVS. Because a certain percentage of women will end up miscarrying at this point in pregnancy anyway, there's no way of knowing for sure whether a miscarriage following CVS was actually caused by the procedure. Your particular risk depends in large part on the skill and experience of the doctor performing the procedure.

Some older studies found that CVS may have caused defects in a baby's fingers or toes, but this was mostly seen in tests done on women before 9 weeks of pregnancy. Current research suggests that there is no increased risk for this problem in women who have CVS at 11 weeks or later.

Who Should Be Tested With CVS?

The American College of Obstetrics and Gyaecology recommends offering CVS when there is an increased risk for a genetic disorder in the baby. This may include:

a. Pregnant women who will be age 35 or older on their due date (the risk of having a baby with a chromosomal problem such as Down syndrome increases with the age of the woman)

b. Couples who already have had a child with a birth defect or have a family history of certain birth defects.

c. Couples with a parent known to carry a chromosomal abnormality or genetic disease

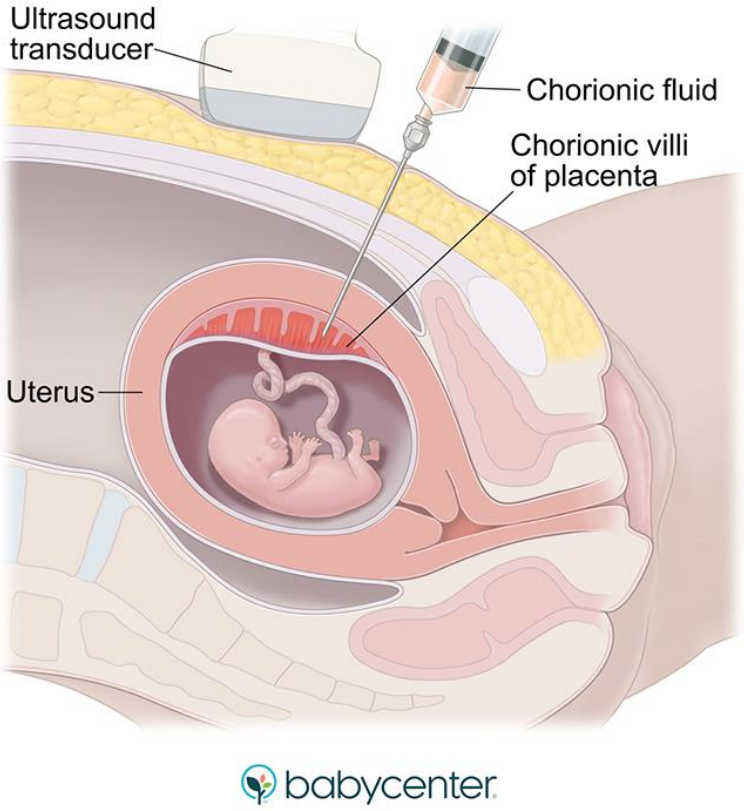
Pregnant women with other abnormal genetic test results

How Is the CVS Test Performed?

Before undergoing a CVS prenatal test, appropriate genetic counselling, including a detailed discussion regarding the risks and benefits of the procedure, are recommended. At the time of initial consultation and counselling, an ultrasound exam will be performed to confirm gestational age (the development stage of the embryo) and the location of the placenta. This is done so that CVS can be performed at the appropriate gestational age (which is usually 10 to 12 weeks from the woman's last menstrual period).There are two ways to collect chorionic villi from the placenta: through the vagina or through the abdomen.

To collect cells through the vagina, a speculum is inserted (in the same way as a Pap test). Then a very thin, plastic tube is inserted up the vagina and into the cervix. Using ultrasound images, the tube is guided up to the placenta, where a small sample is removed.

To collect cells through the abdomen, a slender needle is inserted through the woman's abdomen to the placenta, much like in amniocentesis. The sample of chorionic villi is then sent to a lab, where the cells are grown in a special fluid and tested a few days later. Culture results will be available within two weeks. Your doctor will notify you of the results.



What Happens After the CVS Test?

You'll need to take it easy immediately after a CVS test, so arrange for someone to drive you home. For the rest of the day, you'll need to rest. Generally, women are advised to abstain from strenuous physical activity, sex, and exercise for three days following the procedure. You may have some cramping and bleeding, which is normal, but do tell your doctor or midwife. If you notice fluid leaking from your vagina, call your health care provider immediately.

How would I decide between CVS and amniocentesis?

Both tests can tell you whether your baby has a chromosomal problem or certain genetic disorders. CVS is done earlier in pregnancy (usually between 10 and 13 weeks), so you can find out sooner about your baby's condition. If everything's okay, your mind will be put at ease that much sooner. Or, if there is a serious problem and you opt to terminate the pregnancy, you'll be able to do so while you're still in the first trimester.

On the other hand, you may prefer to wait for the results from second-trimester screening before subjecting yourself to an invasive test. At that point, amniocentesis would be your only option.

Other considerations may influence your decision as well. For example, if you're at high risk for having a baby with a neural tube defect, you may want to have amniocentesis, as CVS cannot diagnose these defects.

CVS is generally thought to have a slightly higher miscarriage rate than amniocentesis, but this may not be the case everywhere. Medical centers that perform a lot of these procedures may have similar miscarriage rates for both. However, only a small percentage of doctors perform CVS, so in some areas it may be difficult to find an experienced specialist who does the procedure.
Women who choose to have CVS or amniocentesis are often those at increased risk for genetic and chromosomal problems, in part because these tests are invasive and carry a small risk of miscarriage. The main advantage of CVS over amniocentesis is that you can have it done earlier — generally between 10 and 13 weeks of pregnancy. For an amnio, you'll have to wait until you're at least 16 weeks pregnant.

C. Ultrasonography:

Definition: An ultrasound scan is a medical test that uses high-frequency sound waves to capture live images from the inside of your body. It's also known as sonography. The technology is similar to that used by sonar and radar, which help the military detect planes and ships. An ultrasound allows your doctor to see problems with organs, vessels, and tissues without needing to make an incision. Unlike other imaging techniques, ultrasound uses no radiation. For this reason, it's the preferred method for viewing a developing foetus during pregnancy.

Why an ultrasound is performed:

Most people associate ultrasound scans with pregnancy. These scans can provide an expectant mother with the first view of her unborn child. However, the test has many other uses. Doctor may order an ultrasound if you're having pain, swelling, or other symptoms that require an internal view of your organs. An ultrasound can provide a view of the:

- bladder
- brain (in infants)
- eyes
- gallbladder
- kidneys
- liver
- ovaries
- pancreas
- spleen
- thyroid
- testicles
- uterus
- blood vessels

An ultrasound is also a helpful way to guide surgeons' movements during certain medical procedures, such as biopsies.

How to prepare for an ultrasound

The steps you will take to prepare for an ultrasound will depend on the area or organ that is being examined. Your doctor may tell you to fast for eight to 12 hours before your ultrasound, especially if your abdomen is being examined. Undigested food can block the sound waves, making it difficult for the technician to get a clear picture.

For an examination of the gallbladder, liver, pancreas, or spleen, you may be told to eat a fat-free meal the evening before your test and then to fast until the procedure. However, you can continue to drink water and take any medications as instructed. For other examinations, you may be asked to drink a lot of water and to hold your urine so that your bladder is full and better visualized. Be sure to tell your doctor about any prescription drugs, over-the-counter medications, or herbal supplements that you take before the exam.

It's important to follow your doctor's instructions and ask any questions you may have before the procedure. An ultrasound carries minimal risks. Unlike X-rays or CT scans, ultrasounds use no radiation. For this reason, they are the preferred method for examining a developing foetus during pregnancy.

How an ultrasound is performed:

Before the exam, you will change into a hospital gown. You will most likely be lying down on a table with a section of your body exposed for the test. An ultrasound technician, called a sonographer, will apply a special lubricating jelly to your skin. This prevents friction so they can rub the ultrasound transducer on your skin. The transducer has a similar appearance to a microphone. The jelly also helps transmit the sound waves.

The transducer sends high-frequency sound waves through your body. The waves echo as they hit a dense object, such as an organ or bone. Those echoes are then reflected back into a computer. The sound waves are at too high of a pitch for the human ear to hear. They form a picture that can be interpreted by the doctor. Depending on the area being examined, you may need to change positions so the technician can have better access.

After the procedure, the gel will be cleaned off of your skin. The whole procedure typically lasts less than 30 minutes, depending on the area being examined. You will be free to go about your normal activities after the procedure has finished.

After an ultrasound:

Following the exam, your doctor will review the images and check for any abnormalities. They will call you to discuss the findings, or to schedule a follow-up appointment. Should anything abnormal turn up on the ultrasound, you may need to undergo other diagnostic techniques, such as a CT scan, MRI, or a biopsy sample of tissue depending on the area examined. If your doctor is able to make a diagnosis of your condition based on your ultrasound, they may begin your treatment immediately.

Probable Questions:

1. Discuss the causes of Down Syndrome.
2. How Down syndrome can be diagnosed before birth?
3. How Down syndrome can be diagnosed after birth?
4. Discuss about prognosis of Down Syndrome?
5. Discuss about epidemiology of Down syndrome.
6. What are the causes of Fragile X syndrome?
7. Discuss about signs and symptoms of Fragile X syndrome.
8. Discuss epidemiology of Fragile X syndrome.
9. Discuss prognosis of Fragile X syndrome.
10. What are the pathophysiology of Fragile X syndrome.
11. Discuss about diagnosis of Fragile X syndrome.
12. Define amniocentesis? How it is done?
13. Describe the method of amniocentesis?
14. Discuss the risks of amniocentesis.
15. Define chorionic villus sampling.
16. What are the benefits of chorionic villus sampling.
17. What kind of problem chorionic villus sampling can identify?
18. What are the risks of chorionic villus sampling?
19. How chorionic villus sampling is done?
20. Compare chorionic villus sampling and amniocentesis.
21. What are the utility of ultrasonography?
22. How an ultrasound is performed ?

Suggested Readings:

1. Principles of Genetics. Snustad and Simmons.
2. Genetics . Verma and Agarwal.
3. Principles of Genetics by Tamarin.
4. Biotechnology by V. Kumaresan
5. Embryology by N. Kumarsen

6. Developmental Biology by Veerbala Rastogi.
7. Embryology by M.P. Arora
8. Developmental Biology by Gilbert.
9 http://www.healthofchildren.com/A/Amniocentesis.html#ixzz6HsOA9h3x

Unit-IV

Future of medicine: Differentiation therapy, gene therapy (Ex Vivo and In vivo), germ line gene therapy

Objective: In this unit we will discuss about Future of medicines which includes differentiation therapy, ex vivo and in vivo gee therapy and also germ line gene therapy.

Differentiation therapy: An approach to the treatment of advanced or aggressive malignancies in which the malignant cells are treated so that they can resume the process of maturation and differentiation into mature cells. Differentiation therapy is based on the concept that cancer cells are normal cells that have been arrested at or have gone back to an immature or less differentiated state, lack the ability to control their own growth, and so multiply at an abnormally fast rate. Differentiation therapy aims to force the cancer cell to resume the process of maturation. Although differentiation therapy does not destroy the cancer cells, it restrains their growth and allows the application of more conventional therapies (such as chemotherapy) to eradicate the malignant cells. Differentiation agents tend to have less toxicity than conventional cancer treatments.

The first differentiation agent found to be successful was all-trans-retinoic acid (ATRA) in the treatment of acute promyelocytic leukaemia (APL). APL is the result of a translocation (an exchange of chromosome material) between chromosomes 15 and 17. There are two chromosome breaks: one in chromosome 15 and the other in chromosome 17. The break in chromosome 15 disrupts the promyelocytic leukaemia (PML) gene which encodes a growth suppressing transcription factor. And the break in chromosome 17 interrupts the retinoic acid receptor alpha (RARa) gene which regulates myeloid differentiation. The translocation creates a PML/RARa fusion gene. It produces an abnormal protein referred to as a chimeric protein that causes an arrest of maturation in myeloid cell maturation at the promyelocytic stage. (It reduces terminal cell differentiation.) And this causes the increased proliferation of promyelocytes.

Traditional chemotherapy or radiotherapy generally involves killing tumour cells . However, cancer cells may instead be coaxed into becoming normal cells by differentiation therapy, which aims to reactivate endogenous differentiation programs in cancer cells to resume the maturation process and eliminate tumour phenotypes (Figure below). Generally, differentiation agents tend to have less toxicity than conventional cancer treatments. A prototype differentiation therapy is all-trans-retinoic acid (ATRA), which induces complete remission in patients with acute promyelocytic leukaemia (APL). ATRA induces terminal cell differentiation by disrupting the promyelocytic leukaemia/retinoic acid receptor α (PML/RARα) fusion protein that arrests the maturation of myeloid cells at the promyelocytic stage. Subsequently, emerging studies have focused on elucidating the mechanisms of action of differentiation therapy in cancers, particularly in solid tumours.

Most APL patients are now treated first with all-trans-retinoic acid (ATRA). It causes the promyelocytes to differentiate (to mature) and so deters them from proliferating. ATRA induces a complete remission in about 70% of cases. ATRA is the prototype of a differentiation therapy agent.

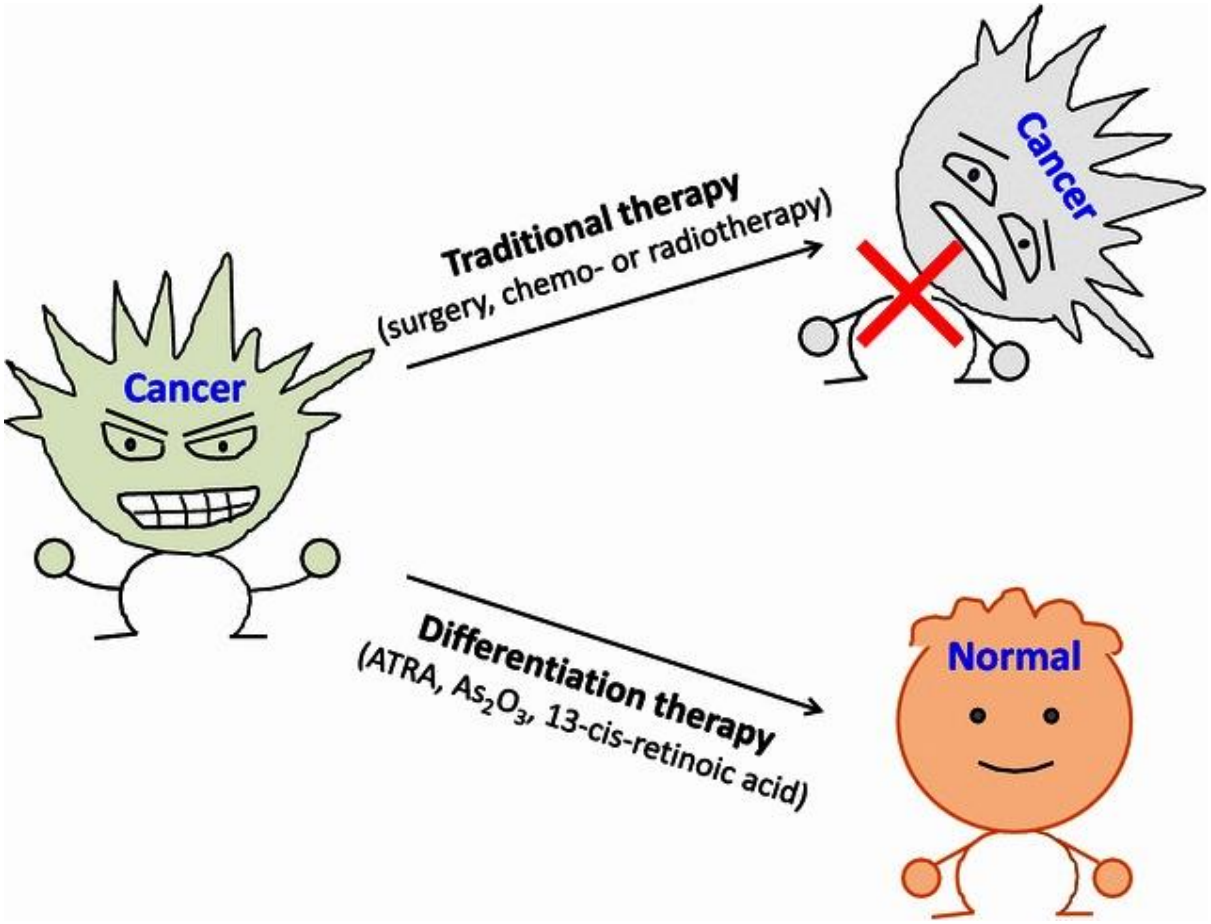


Figure: Diagram of differentiation therapy. Compared with traditional cancer treatments, such as surgery, chemotherapy, and radiotherapy that aim to kill tumour cells, differentiation therapy has opened a new door for the treatment of malignant tumours. Differentiation therapy is based on the concept that a neoplasm is a differentiation disorder or a dedifferentiation disease. In response to the induction of differentiation, tumour cells can revert to normal or nearly normal cells, thereby altering their malignant phenotype and ultimately alleviating the tumour burden or curing the malignant disease without damaging normal cells. ATRA, all-trans-retinoic acid

Gene Therapy:

Gene therapy is a novel treatment method which utilizes genes or short oligonucleotide sequences as therapeutic molecules, instead of conventional drug compounds. This technique is widely used to treat those defective genes which contribute to disease development. Gene therapy involves the introduction of one or more foreign genes into an organism to treat hereditary or acquired genetic defects. In gene therapy, DNA encoding a therapeutic protein is

packaged within a "vector", which transports the DNA inside cells within the body. The disease is treated with minimal toxicity, by the expression of the inserted DNA by the cell machinery. In 1990 FDA for the first time approved a gene therapy experiment on ADA-SCID in the United States after the treatment of Ashanti DeSilva. After that, approximately 1700 clinical trials on patients have been performed with various techniques and genes for numerous diseases. Gene therapy is the process of inserting genes into cells to treat diseases. The newly introduced genes will encode proteins and correct the deficiencies that occur in genetic diseases. Thus, gene therapy primarily involves genetic manipulations in animals or humans to correct a disease, and keep the organism in good health. The initial experiments on gene therapy are carried out in animals, and then in humans. Obviously, the goal of the researchers is to benefit the mankind and improve their health.

An overview of gene therapy strategies is depicted in Fig. 13.1. In gene augmentation therapy, a DNA is inserted into the genome to replace the missing gene product. In case of gene inhibition therapy, the antisense gene inhibits the expression of the dominant gene.

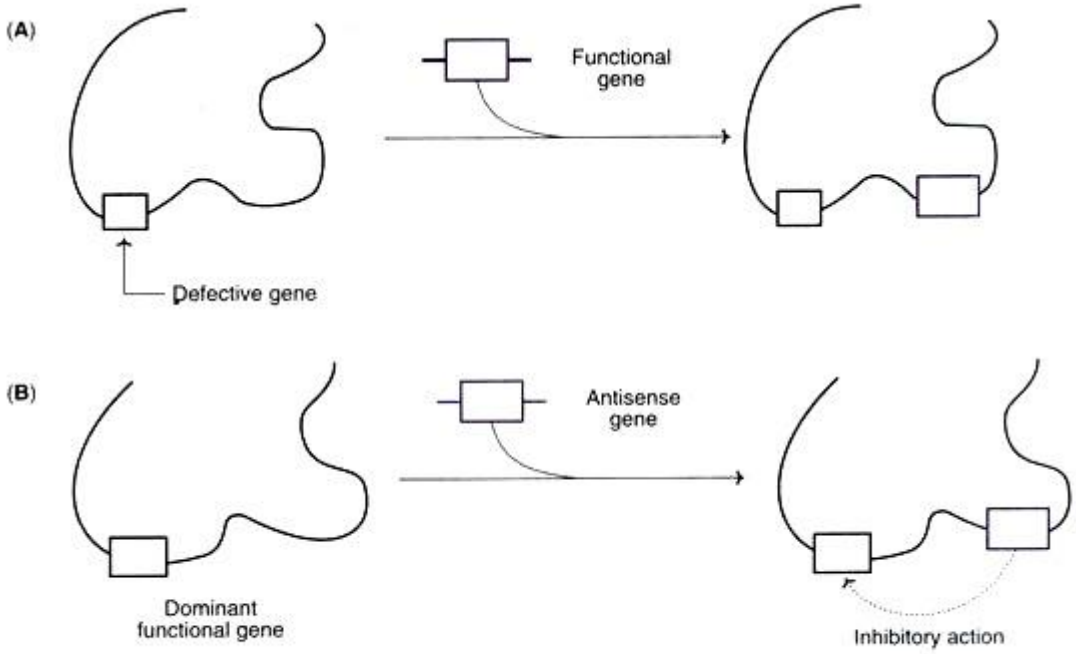


***Fig. 13.1** : Overview of two major gene therapy strategies*
(A) Gene augmentation therapy (B) Gene inhibition therapy.

I. General gene therapy strategies

a. Gene Augmentation Therapy (GAT):

For diseases caused by loss of function of a gene, introducing extra copies of the normal gene may increase the amount of normal gene product to a level where the normal phenotype is restored (see Fig. 23.1). As a result GAT is targeted at clinical disorders where the pathogenesis is reversible. It also helps to have no precise requirement for expression levels of the introduced gene and a clinical response at low expression levels. GAT has been particularly applied to autosomal recessive disorders where even modest expression levels of an introduced gene may make a substantial difference.

Dominantly inherited disorders are much less amendable to treatment; gain-of-function mutations are not treatable by this approach and, even if there is a loss-of- function mutation, high expression efficiency of the introduced gene is required: individuals with 50% of normal gene product are normally affected, and so the challenge is to increase the amount of gene product towards normal levels.

b. Targeted Killing of Specific Cells:

This general approach is popular in cancer gene therapies. Genes are directed to the target cells and then expressed so as to cause cell killing. Direct cell killing is possible if the inserted genes are expressed to produce a lethal toxin (suicide genes), or a gene encoding a pro drug is inserted, conferring susceptibility to killing by a subsequently administered drug. Indirect cell killing uses immunostimulatory genes to provoke or enhance an immune response against the target cell.

c. Targeted Mutation Correction:

If an inherited mutation produces a dominant-negative effect, gene augmentation is unlikely to help. Instead, the resident mutation must be corrected. Because of practical difficulties, this approach has yet to be applied but, in principle, it can be done at different levels: at the gene level (e.g. by gene targeting methods based on homologous recombination); or at the RNA transcript level (e.g. by using particular types of therapeutic ribozymes — or therapeutic RNA editing).

d. Targeted Inhibition of Gene Expression:

If disease cells display a novel gene product or inappropriate expression of a gene (as in the case of many cancers, infectious diseases, etc.), a variety of different systems can be used specifically to block the expression of a single gene at the DNA, RNA or protein levels. Allele-specific inhibition of expression may be possible in some cases, permitting therapies for some disorders resulting from dominant negative effects.

II. Approaches for Gene Therapy:

There are two approaches to achieve gene therapy.

1. Somatic Cell Gene Therapy:

The non- reproductive (non-sex) cells of an organism are referred to as somatic cells. These are the cells of an organism other than sperm or eggs cells, e.g., bone marrow cells, blood cells, skin cells, intestinal cells. At present, all the research on gene therapy is directed to correct the genetic defects in somatic cells. In essence, somatic cell gene therapy involves the insertion of a fully functional and expressible gene into a target somatic cell to correct a genetic disease permanently.

2. Germ Cell Gene Therapy:

The reproductive (sex) cells of an organism constitute germ cell line. Gene therapy involving the introduction of DNA into germ cells is passed on to the successive generations. For safety, ethical and technical reasons, germ cell gene therapy is not being attempted at present.

The genetic alterations in somatic cells are not carried to the next generations. Therefore, somatic cell gene therapy is preferred and extensively studied with an ultimate objective of correcting human diseases. Development of gene therapy in humans for any specific disease involves the following steps. In fact, this is a general format for introducing any therapeutic agent for human use.

a. In vitro experiments and research on laboratory animals (pre-clinical trials).
b. Phase I trials with a small number (5-10) of human subjects to test safety of the product.
c. Phase II trials with more human subjects to assess whether the product is helpful.
d. Phase III trials in large human samples for a final and comprehensive analysis of the safety and efficacy of the product.

As such, gene therapy involves a great risk. There are several regulatory agencies whose permission must be sought before undertaking any work related to gene therapy. Recombinant DNA Advisory Committee (RAC) is the supervisory body of the National Institute of Health, U.S.A., that clears proposals on experiments involving gene therapy. A large number of genetic disorders and other diseases are currently at various stages of gene therapy trials. A selected list of some important ones is given in Table 13.1.

TABLE 13.1 Human gene therapy trials

Disease	*Gene therapy*
Severe combined immunodeficiency (SCID)	Adenosine deaminase (ADA).
Cystic fibrosis	Cystic fibrosis transmembrane regulator (CFTR).
Familial hypercholesterolemia	Low density lipoprotein (LDL) receptor.
Emphysema	α_1-Antitrypsin
Hemophilia B	Factor IX
Thalassemia	α- or β-Globin
Sickle-cell anemia	β-Globin
Lesch-Nyhan syndrome	Hypoxanthine-guanine phosphoribosyltransferase (HGPRT).
Gaucher's disease	Glucocerebrosidase
Peripheral artery disease	Vascular endothelial growth factor (VEGF)
Fanconi anemia	Fanconi anemia C
Melanoma	Tumor necrosis factor (TNF)
Melanoma, renal cancer	Interleukin-2 (IL-2)
Glioblastoma (brain tumor), AIDS, ovarian cancer	Thymidine kinase (herpes simplex virus)
Head and neck cancer	p^{53}
Breast cancer	Multidrug resistance I
AIDS	*rev* and *env*
Colorectal cancer, melanoma, renal cancer	Histocompatability locus antigen-B_7 (HLA-B_7)
Duchenne muscular dystrophy	Dystrophin
Short stature*	Growth hormone
Diabetes*	Glucose transporter-2, (GLUT-2), glucokinase
Phenylketonuria*	Phenylalanine hydroxylase
Citrullinemia*	Arginosuccinate synthetase

III. Methods of gene therapy:

There are mainly two approaches for the transfer of genes in gene therapy:

1. Transfer of genes into patient cells outside the body (ex vivo gene therapy)

2. Transfer of genes directly to cells inside the body (in vivo).

1. Ex vivo gene therapy:

The ex vivo gene therapy can be applied to only selected tissues (e.g., bone marrow) whose cells can be cultured in the laboratory. The technique of ex vivo gene therapy involves the following steps (Fig. 13.2).

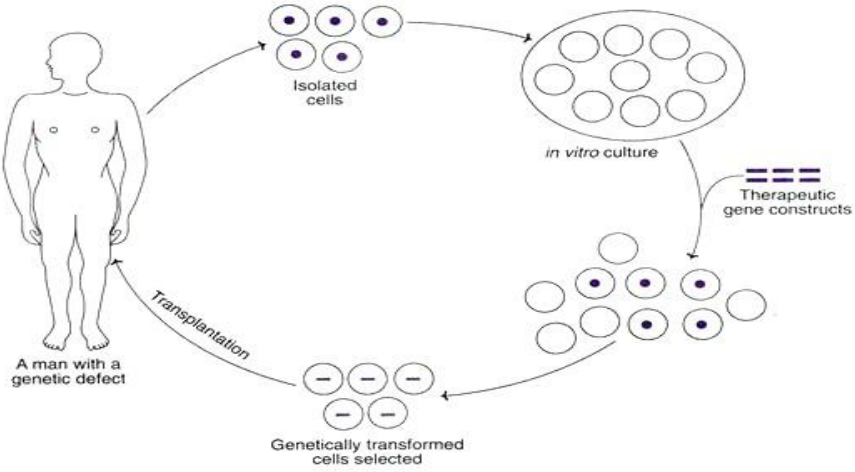


Fig. 13.2 : The procedure for ex vivo gene therapy.

1. Isolate cells with genetic defect from a patient.
2. Grow the cells in culture.
3. Introduce the therapeutic gene to correct gene defect.
4. Select the genetically corrected cells (stable trans-formants) and grow.
5. Transplant the modified cells to the patient.

The procedure basically involves the use of the patient's own cells for culture and genetic correction, and then their return back to the patient. This technique is therefore, not associated with adverse immunological responses after transplanting the cells. Ex vivo gene therapy is efficient only, if the therapeutic gene (remedial gene) is stably incorporated and continuously expressed. This can be achieved by use of vectors.

Vectors in Gene Therapy:
The carrier particles or molecules used to deliver genes to somatic cells are referred to as vectors. The important vectors employed in ex vivo gene therapy are listed below and briefly described next.

i. Viruses

ii. Human artificial chromosome

iii. Bone marrow cells.

i. Viruses:
The vectors frequently used in gene therapy are viruses, particularly retroviruses. RNA is the genetic material in retroviruses. As the retrovirus enters the host cell, it synthesizes DNA from RNA (by reverse transcription). The so formed viral DNA (referred to as provirus) gets incorporated into the DNA of the host cell.

The proviruses are normally harmless. However, there is a tremendous risk, since some of the retroviruses can convert normal cells into cancerous ones. Therefore, it is absolutely essential to ensure that such a thing does not happen.

Making retroviruses harmless:

Researchers employ certain biochemical methods to convert harmful retroviruses to harmless ones, before using them as vectors. For instance, by artificially removing a gene that encodes for the viral envelope, the retrovirus can be crippled and made harmless. This is because, without the envelope, retrovirus cannot enter the host cell. The production of a large number (billions) of viral particles can be achieved, starting from a single envelope defective retrovirus (Fig. 13.3).

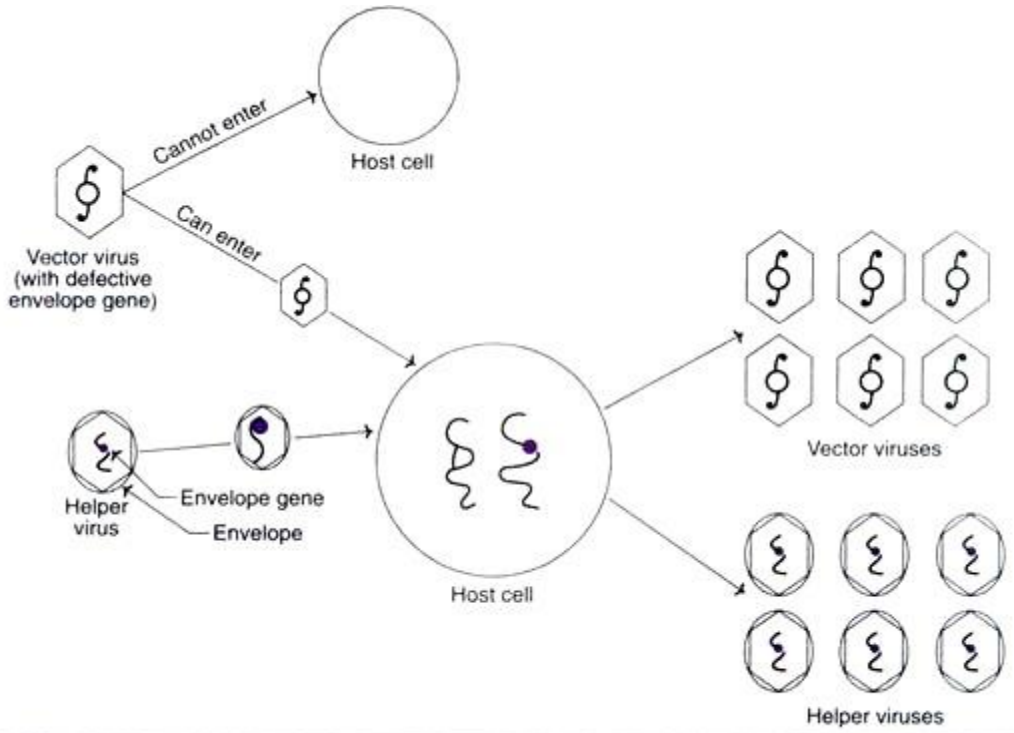


Fig. 13.3 : Large scale production of vector viruses by using helper viruses.

This is made possible by using helper viruses which contain normal gene for envelope formation. Along with the helper virus, the vector (with defective envelope gene) can enter the host cell and both of them multiply. By repeated multiplication in host cells, billions of vector and helper viruses are produced.

The vector viruses can be separated from the helper viruses and purified. Isolation of vector viruses, totally free from helper viruses, is absolutely essential. Contamination of helper viruses is a big threat to the health of the patients undergoing gene therapy.

Retroviruses in gene therapy:

The genetic map of a typical retrovirus is depicted in Fig. 13.4A. In general, the retrovirus particle has RNA as a genome organized into six regions. It has a 5′-long terminal repeat (5′-LTR), a non-coding sequence required for packaging RNA designated as psi (Ψ), a gene gag

coding for structural protein, a gene pol that codes for reverse transcriptase, a gene env coding for envelope protein and a 3-LTR sequence.

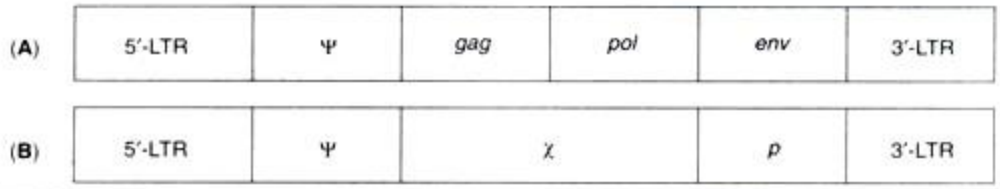


Fig. 13.4 : A retrovirus used in gene therapy. (A) General map of a typical retrovirus (B) Gene map of a modified retrovirus for use in gene therapy (LTR-Long terminal repeat; Ψ-Packaging signal sequence; gag-*Coding sequence for structural protein;* pol-*Coding sequence for reverse transcriptase;* env-*Envelope protein coding sequence; χ-Therapeutic gene; p–Promoter gene).*

For use of a retrovirus as a vector, the structural genes gag and pol are deleted. These genes are actually adjacent to Ψ region. In addition, a promoter gene is also included (Fig. 13.4B). This vector design allows the synthesis of cloned genes. A retroviral vector can carry a therapeutic DNA of maximum size of 8 kb.

A retroviral vector DNA can be used to transform the cells. However, the efficiency of delivery and integration of therapeutic DNA are very low. In recent years, techniques have been developed to deliver the vector RNA to host cells at a high frequency. For this purposes, packaged retroviral RNA particles are used. This technique allows a high efficiency of integration of pharmaceutical DNA into host genome.

Several modified viral vectors have been developed in recent years for gene therapy. These include onco-retrovirus, adenovirus, adeno-associated virus, herpes virus and a number of hybrid vectors combining the good characters of the parental vectors.

Murine leukaemia viruses in gene therapy:

This is a retrovirus that causes a type of leukaemia in mice. It can react with human cells as well as the mouse cells, due to a similarity in the surface receptor protein. Murine leukaemia virus (MLV) is frequently used in gene transfer.

AIDS virus in gene therapy?

It is suggested that the human immunodeficiency virus (HIV) can be used as a vector in gene transfer. But this is bound to create public uproar. Some workers have been successful in creating a harmless HIV (crippled HIV) by removing all the genes related to reproduction. At the same time, the essential genes required for gene transfer are retained. There is a distinct advantage with HIV when compared with MLV. MLV is capable of bringing out gene transfer only in dividing cells. HIV can infect even non-dividing cells (e.g., brain cells) and do the job of gene transfer effectively. However, it is doubtful whether HIV can ever be used as a vector.

ii. Human Artificial Chromosome:

The details of human artificial chromosome (HAC) are described elsewhere .HAC is a synthetic chromosome that can replicate with other chromosomes, besides encoding a human protein. As already discussed above, use of retroviruses as vectors in gene therapy is associated with a heavy risk. This problem can be overcome if HAC is used. Some success has been achieved in this direction.

iii. Bone Marrow Cells:

Bone marrow contains totipotent embryonic stem (ES) cells. These cells are capable of dividing and differentiating into various cell types (e.g., red blood cells, platelets, macrophages, osteoclasts, B- and T-lymphocytes). For this reason, bone marrow transplantation is the most widely used technique for several genetic diseases.

And there is every reason to believe that the genetic disorders that respond to bone marrow transplantation are likely to respond to ex vivo gene therapy also (Table 13.2). For instance, if there is a gene mutation that interferes with the function of erythrocytes (e.g., sickle-cell anaemia), bone marrow transplantation is done. Bone marrow cells are the potential candidates for gene therapy of sickle-cell anaemia. However, this is not as simple as theoretically stated.

TABLE 13.2 Selected list of genetic diseases that are likely to be cured by using bone marrow cells (potential candidates for gene therapy)

Severe combined immunodeficiency (SCID)
Sickle-cell anemia
Fanconi anemia
Thalassemia
Gaucher's disease
Hunter disease
Hurler syndrome
Chronic granulomatous disease
Infantile agranulocytosis
Osteoporosis
X-linked agammaglobulinemia

Selected Examples of Ex Vivo Gene Therapy:

a. Therapy for Adenosine Deaminase Deficiency:

The first and the most publicized human gene therapy was carried out to correct the deficiency of the enzyme adenosine deaminase (ADA). This was done on September 14, 1990 by a team of workers led by Blaese and Anderson at the National Institute of Health, USA (The girl's name is Ashanti, 4 years old then).

b. Severe combined immunodeficiency (SCID):

This is rare inherited immune disorder associated with T-lymphocytes, and (to a lesser extent) B-lymphocytes dysfunction. About 50% of SCID patients have a defect in the gene (located on chromosome 20, and has 32,000 base pairs and 12 exons) that encodes for adenosine deaminase. In the deficiency of ADA, deoxyadenosine and its metabolites (primarily deoxyadenosine 5′-triphosphate) accumulate and destroy T-lymphocytes.

T-Lymphocytes are essential for body's immunity. Besides participating directly in body's defence, they promote the function of B-lymphocytes to produce antibodies. Thus, the patients of SCID (lacking ADA) suffer from infectious diseases and die at an young age. Previously,

the children suffering from SCID were treated with conjugated bovine ADA, or by bone marrow transplantation.

c. Technique of therapy for ADA deficiency:

The general scheme of gene therapy adopted for introducing a defective gene in the patient has been depicted in Fig 13.2. The same procedure with suitable modifications can also be applied for other gene therapies.

A plasmid vector bearing a pro-viral DNA is selected. A part of the pro-viral DNA is replaced by the ADA gene and a gene (G 418) coding for antibiotic resistance, and then cloned. The antibiotic resistance gene will help to select the desired clones with ADA gene.

A diagrammatic representation of the treatment of ADP deficient patient is depicted in Fig. 13.5.

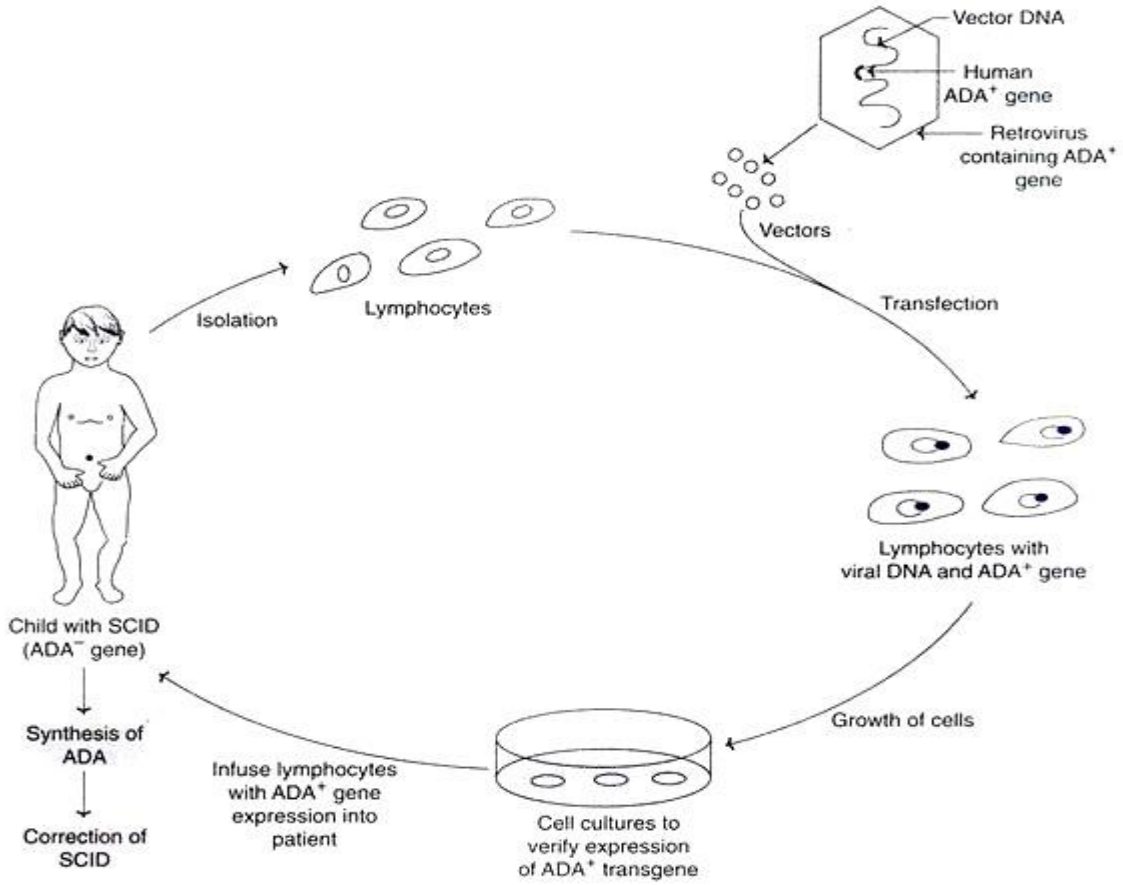


Fig. 13.5 : Treatment of adenosine deaminase (ADA) deficient patient by somatic ex vivo gene therapy (SCID-Severe combined immunodeficiency disease).

Circulating lymphocytes are removed from a patient suffering from ADA deficiency. These cells are transfected with ADA gene by exposing to billions of retroviruses carrying the said gene. The genetically-modified lymphocytes are grown in cultures to confirm the expression of ADA gene and returned to the patient. These lymphocytes persist in the circulation and synthesize ADA.

Consequently, the ability of the patient to produce antibodies is increased. However, there is a limitation. The lymphocytes have a short life span (just live for a few months), hence the transfusions have to be carried out frequently.

Transfer of ADA gene into stem cells:
In 1995, ADA gene was transferred into the stem cells, obtained from the umbilical cord blood, at the time of baby's delivery. Four days after birth, the infant received the modified cells back. By this way, a permanent population of ADA gene producing cells was established.

d. Therapy for Familial Hypercholesterolemia:
The patients of familial hypercholesterolemia lack the low density lipoprotein (LDL) receptors on their liver cells. As a result, LDL cholesterol is not metabolised in liver. The accumulated LDL- cholesterol builds up in the circulation, leading to arterial blockage and heart diseases.
Attempts are being made by gene therapists to help the victims of familial hypercholesterolemia. In fact, there is some success also. In a woman, 15% of the liver was removed. The hepatocytes were transduced with retroviruses carrying genes for LDL receptors. These genetically modified hepatocytes were infused into the patient's liver.
The hepatocytes established themselves in the liver and produced functional LDL-receptors. A significant improvement in the patient's condition, as assessed by estimating the lipid parameters in blood, was observed. Further, there were no antibodies produced against the LDL-receptor molecules, clearly showing that the genetically modified liver cells were accepted.

e. Therapy for Lesch-Nyhan Syndrome:
Lesch-Nyhan syndrome is an inborn error in purine metabolism due to a defect in a gene that encodes for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HCPRT). In the absence of HGPRT, purine metabolism is disturbed and uric acid level builds up, resulting in severe gout and kidney damage. The victims of Lesch- Nyhan syndrome exhibit symptoms of mental retardation, besides an urge to bite lips and fingers, causing self-mutilation.
By using retroviral vector system, HGPRT producing genes were successfully inserted into cultured human bone marrow cells. The major problem in humans is the involvement of brain. Experiments conducted in animals are encouraging. However, it is doubtful whether good success can be achieved by gene therapy for Lesch-Nyhan syndrome in humans, in the near future.

f. Therapy for Haemophilia:
Haemophilia is a genetic disease due lack of a gene that encodes for clotting factor IX. It is characterized by excessive bleeding. By using a retroviral vector system, genes for the synthesis of factor IX were inserted into the liver cells of dogs. These dogs no longer displayed the symptoms of haemophilia.

g. Ex Vivo Gene Therapy with Non-Autologous Cells:
The ex vivo gene therapies described above are based on the transplantation of genetically modified cells for the production of desired proteins. However, there are several limitations in

using the patient's own cells (autologous cells) for gene therapy. These include lack of enough cells from target tissues, defective uptake of genes and their inadequate expression. To overcome these problems, attempts are on to develop methods to use non-autologous cells (i.e., cells from other individuals or animals). The outline of the procedure is briefly described below.

Tissue-specific cells capable of growing in culture are selected. These include fibroblasts from skin, hepatocytes from liver, and myoblasts from muscle and astrocytes from brain. These cells are cultured and genetically modified with the therapeutic gene. They are then encapsulated in artificial membrane composed of a synthetic polymer (e.g., polyether sultone, alginase-poly L-lysine-alginate).The polymeric membranes are non-immunogenic, therefore the patient can accept non-autologous encapsulated cells. Further, being semipermeable in nature, these membranes allow the nutrients to enter in, and the encoded protein (by the therapeutic gene) to pass out.

Experiments conducted in animals have shown some encouraging results for using non-autologous cells in gene therapy. The encapsulated cells were found to proliferate and produce the required protein. However, the success has been very limited in human trials.

2. In Vivo Gene Therapy:

The direct delivery of the therapeutic gene (DNA) into the target cells of a particular tissue of a patient constitutes in vivo gene therapy (Fig. 13.6). Many tissues are the potential candidates for this approach. These include liver, muscle, skin, spleen, lung, brain and blood cells. Gene delivery can be carried out by viral or non- viral vector systems. The success of in vivo gene therapy mostly depends on the following parameters

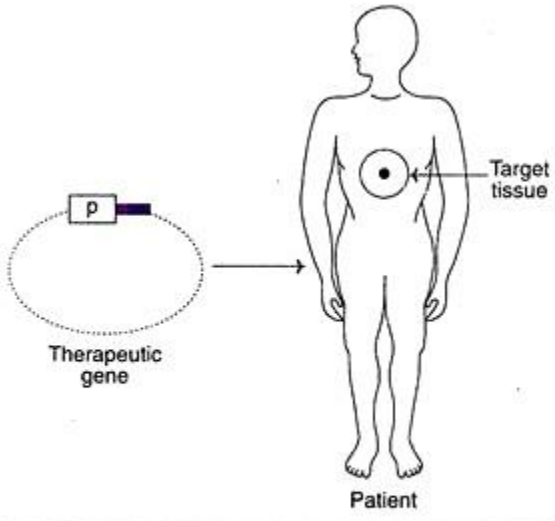


Fig. 13.6 : Diagrammatic representation of in vivo *gene therapy. (p-Promoter gene specific for therapeutic gene)*

i. The efficiency of the uptake of the remedial (therapeutic) gene by the target cells.
ii. Intracellular degradation of the gene and its uptake by nucleus.
iii. The expression capability of the gene.

In vivo gene therapy with special reference to gene delivery systems (viral, non-viral) with suitable examples is described.

Gene Delivery by Viruses:

Many viral vector systems have been developed for gene delivery. These include retroviruses, adenoviruses, adeno-associated viruses and herpes simplex virus.

Retrovirus vector system:

Replication defective retrovirus vectors that are harmless are being used. A plasmid in association with a retrovirus, a therapeutic gene and a promoter is referred to as plasmovirus. The plasmovirus is capable of carrying a DNA (therapeutic gene) of size less than 3.4 kb. Replication defective virus particles can be produced from the plasmovirus.

As such, for the delivery of genes by retroviral vectors, the target cells must be in a dividing stage. But majority of the body cells are quiescent. In recent years, viral vectors have been engineered to infect non-dividing cells. Further, attempts are on to include a DNA in the retroviral vectors (by engineering env gene) that encodes for cell receptor protein. If this is successfully achieved, the retroviral vector will specifically infect the target tissues.

Adenoviral vector system:

Adenoviruses (with a DNA genome) are considered to be good vectors for gene delivery because they can infect most of the non-dividing human cells. A common cold adenovirus is a frequently used vector. As the target cells are infected with a recombinant adenovirus, the therapeutic gene (DNA) enters the nucleus and expresses itself.

However, this DNA does not integrate into the host genome. Consequently, adenoviral based gene therapy required periodic administration of recombinant viruses. The efficiency of gene delivery by adenoviruses can be enhanced by developing a virus that can specifically infect target cells. This is possible by incorporating a DNA encoding a cell receptor protein.

Adeno-associated virus vector system:

Adeno-associated virus is a human virus that can integrate into chromosome 19. It is a single-stranded, non-pathogenic small DNA virus (4.7 kb). As the adeno-associated virus enters the host cell, the DNA becomes double- stranded, gets integrated into chromosome and expresses. Adeno-associated viruses can serve as good vectors for the delivery of therapeutic genes. Recombinant viruses are created by using two plasmids and an adenovirus (i.e., helper virus) by a special technique. Some attempts were made to use therapeutic genes for the treatment of the human diseases-haemophilia (for production of blood clotting factor IX) and cystic fibrosis (for synthesis of cystic fibrosis trans membrane regulator protein) by employing adeno-associated viruses.

Therapy for cystic fibrosis:

Cystic fibrosis (CF) is one of the most common (frequency 1: 2,500) and fatal genetic diseases. It is characterized by the accumulation of sticky, dehydrated mucus in the respiratory tract and

lungs. Patients of CF are highly susceptible to bacterial infections in their lungs and most of them die before reaching the age of thirty.

Biochemical basis:

In the normal persons the chloride ions of the cells are pushed out through the participation of a protein called cystic fibrosis trans membrane regulator (CFTR). In the patients of cystic fibrosis, the CFTR protein is not produced due to a gene defect. Consequently, the chloride ions concentrate within the cells which draw water from the surroundings. As a result, the respiratory tract and the lungs become dehydrated with sicky mucus, an ideal environment for bacterial infections.

Gene therapy for Cystic Fibrosis:

As the defective gene for cystic fibrosis was identified in 1989, researchers immediately started working on gene therapy for this disease. Adenoviral vector systems have been used, although the success has been limited. The major drawback is that the benefits are short-lived, since the adenoviruses do not integrate themselves into host cells. Multiple administration of recombinant adenovirus caused immunological responses that destroyed the cells.

By using adeno-associated virus vector system, some encouraging results were reported in the gene therapy of CF. In the phase I clinical trials with CF patients, the vector persisted for about 70 days and some improvement was observed in the patients. Some researchers are trying to insert CF gene into the developing fetal cells (in experimental animals such as mice) to produce CFTR protein. But a major breakthrough is yet to come.

Herpes simplex virus vector system:

The retroviruses and adenoviruses employed in in vivo gene therapy are engineered to infect specific target cells. There are some viruses which have a natural tendency to infect a particular type of cells. The best example is herpes simplex virus (HSV) type I, which infects and persists in non-dividing nerve cells. HSV is a human pathogen that causes (though rarely) cold sores and encephalitis.

These are a large number of diseases (metabolic, neurodegenerative, immunological, tumours) associated with nervous system. HSV is considered as an ideal vector for in vivo gene therapy of many nervous disorders. The HSV has a double-stranded DNA of about 152 kb length as its genome. About 30 kb of HSV genome can be replaced by a cloned DNA without loss of its basic characteristics (replication, infection, packaging etc.). But there are some technical difficulties in dealing with large-sized DNAs in genetic engineering experiments. Some modified HSV vectors with reduced genomic sizes have been developed.

Most of the work on the gene therapy, related to the use of HSV as a vector, is being conducted in experimental animals. And the results are quite encouraging. HSV vectors could deliver therapeutic genes to the brain and other parts of nervous system. These genes are well expressed and maintained for long periods. More research, however, is needed before going for human trials. If successful, HSV may help to treat many neurodegenerative syndromes such as Parkinson’s disease and Alzheimer’s disease by gene therapy.

Gene Delivery by Non-Viral Systems:

There are certain limitations in using viral vectors in gene therapy. In addition to the prohibitive cost of maintaining the viruses, the viral proteins often induce inflammatory responses in the host. Therefore, there is a continuous search by researchers to find alternatives to viral vector systems.

a. Pure DNA constructs:

The direct introduction of pure DNA constructs into the target tissue is quite simple. However, the efficiency of DNA uptake by the cells and its expression are rather low. Consequently, large quantities of DNA have to be injected periodically. The therapeutic genes produce the proteins in the target cells which enter the circulation and often get degraded.

b. Lipoplexes:

The lipid-DNA complexes are referred to as lipoplexes or more commonly liposomes. They have a DNA construct surrounded by artificial lipid layers. A large number of lipoplexes have been prepared and used. They are non-toxic and non-immunogenic.

The major limitation with the use of lipoplexes is that as the DNA is taken up by the cells, most of it gets degraded by the lysosomes. Thus, the efficiency of gene delivery by lipoplex is very low. Some clinical trials using liposome-CFTR gene complex showed that the gene expression was very short-lived.

c. DNA-molecular conjugates:

The use of DNA-molecular conjugates avoids the lysosomal breakdown of DNA. Another advantage of using conjugates is that large-sized therapeutic DNAs (> 10 kb) can be delivered to the target tissues. The most commonly used synthetic conjugate is poly-L-lysine, bound to a specific target cell receptor. The therapeutic DNA is then made to combine with the conjugate to form a complex (Fig. 13.7).

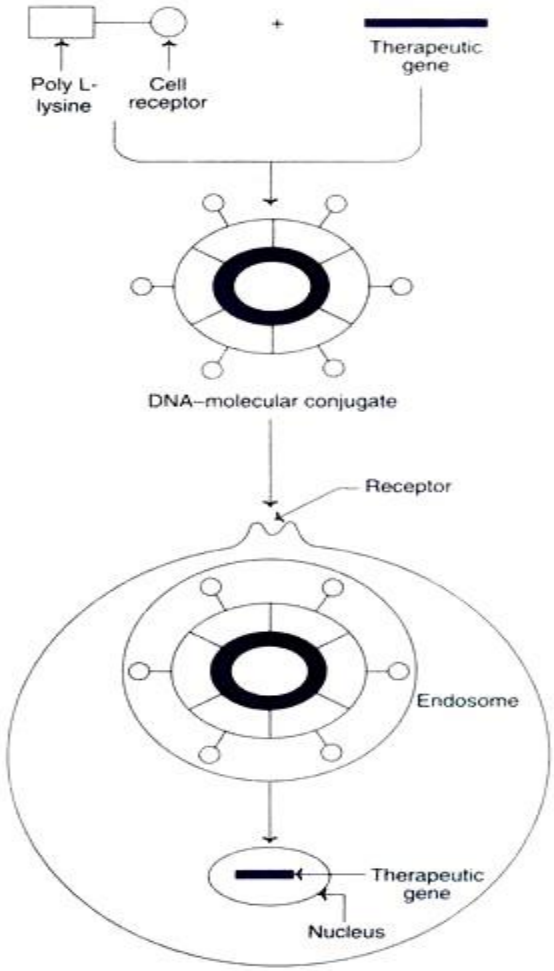


Fig. 13.7 : DNA-molecular conjugate in the delivery of therapeutic gene.

This DNA molecular conjugate binds to specific cell receptor on the target cells. It is engulfed by the cell membrane to form an endosome which protects the DNA from being degraded. The DNA released from the endosome enters the nucleus where the therapeutic gene is expressed.

d. Human artificial chromosome:

Human artificial chromosome (HAC) which can carry a large DNA one or more therapeutic genes with regulatory elements is a good and ideal vector. Studies conducted in cell cultures using HAC are encouraging. But the major problem is the delivery of the large-sized chromosome into the target cells. Researchers are working to produce cells containing genetically engineered HAC. There exists a possibility of encapsulating and implanting these cells in the target tissue. But a long way to go!

Efficiency of gene delivery by non-viral vectors:

Although the efforts are continuously on to find suitable non-viral vectors for gene delivery, the success has been very limited. This is mainly due to the following two reasons.

1. The efficiency of transfection is very low.

2. The expression of the therapeutic gene is for a very short period, consequently there is no effective treatment of the disease.

Gene Therapy Strategies for Cancer:

Cancer is the leading cause of death throughout the world, despite the intensive treatment strategies (surgery, chemotherapy, radiation therapy). Gene therapy is the latest and a new approach for cancer treatment. Some of the developments are briefly described hereunder.

Tumour necrosis factor gene therapy:

Tumour necrosis factor (TNF) is a protein produced by human macrophages. TNF provides defence against cancer cells. This is brought out by enhancing the cancer-fighting ability of tumour- infiltrating lymphocytes (TILs), a special type of immune cells.

The tumour-infiltrating lymphocytes were transformed with a TNF gene (along with a neomycin resistant gene) and used for the treatment of malignant melanoma (a cancer of melanin producing cells usually occurs in skin). TNF as such is highly toxic, and fortunately no toxic side effects were detected in the melanoma patients injected with genetically altered TILs with TNF gene. Some improvement in the cancer patients was observed.

Suicide gene therapy:

The gene encoding the enzyme thymidine kinase is often referred to as suicide gene, and is used for the treatment of certain cancers. Thymidine kinase (TK) phosphorylates nucleosides to form nucleotides which are used for the synthesis of DNA during cell division. The drug ganciclovir (GCV) bears a close structural resemblance to certain nucleosides (thymidine). By mistake, TK phosphorylates ganciclovir to form triphosphate-GCV, a false and unsuitable nucleotide for DNA synthesis. Triphosphate-GCV inhibits DMA polymerase (Fig. 13.8).

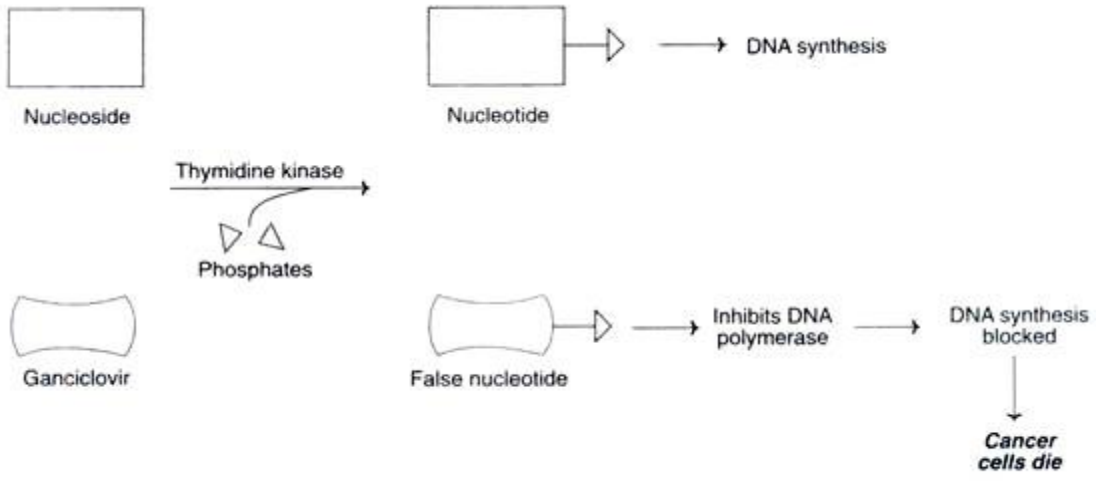


Fig. 13.8 : The action of ganciclovir mediated by thymidine kinase to inhibit the growth of cancer cells.

The result is that the elongation of the DNA molecule abruptly stops at a point containing the false nucleotide (of ganciclovir). Further, the triphosphate-GCV can enter and kill the neighbouring cancer cells, a phenomenon referred to as bystander effect. The ultimate result is that the cancer cells cannot multiply, and therefore die. Thus, the drug ganciclovir can be used to kill the cancer cells.

Ganciclovir is frequently referred to as a pro-drug and this type of approach is called pro-drug activation gene therapy. Ganciclovir has been used for treatment of brain tumours (e.g., glioblastoma, a cancer of glial cells in brain), although with a limited success.

In the suicide gene therapy, the vector used is herpes simplex virus (HSV) with a gene for thymidine kinase (TK) inserted in its genome. Normal brain cells do not divide while the brain tumour cells go on dividing unchecked. Thus, there is a continuous DNA replication in tumour cells. By using GCV-HSVTK suicide gene therapy, some reduction in proliferating tumour cells was reported. Several new strategies are being developed to increase the delivery of HSVTK gene to all the cells throughout a tumour.

Two-gene cancer therapy:

For treatment of certain cancers, two gene systems are put together and used. For instance, TK suicide gene (i.e., GCV-HSVTK) is clubbed with interleukin-2 gene (i.e. a gene promoting immunotherapy). Interleukin-2 produced mobilizes immune response. It is believed that certain proteins are released from the tumour cells on their death.

These proteins, in association with immune cells, reach the tumour and initiate immunological reactions directed against the cancer cells. Two-gene therapies have been carried out in experimental animals with colon cancer and liver cancer, and the results are encouraging.

Gene replacement therapy:

A gene named p^{53} codes for a protein with a molecular weight of 53 kilo Daltons (hence p^{53}). p^{53} is considered to be a tumour-suppressor gene, since the protein it encodes binds with DNA and inhibits replication. The tumour cells of several tissues (breast, brain, lung, skin, bladder, colon, bone) were found to have altered genes of p^{53}(mutated p^{53}), synthesizing different proteins from the original.

These altered proteins cannot inhibit DNA replication. It is believed that the damaged p^{53} gene may be a causative factor in tumour development. Some workers have tried to replace the damaged p^{53}gene by a normal gene by employing adenovirus vector systems .There are some encouraging results in the patients with liver cancer.

Gene Therapy for Aids:

AIDS is a global disease with an alarming increase in the incidence every year. It is invariably fatal, since there is no cure. Attempts are being made to relieve the effects of AIDS by gene therapy. Some of the approaches are discussed hereunder.

a. rev and env genes:

A mutant strain of human immunodeficiency virus (HIV), lacking rev and env genes has been developed. The regulatory and envelope proteins of HIV are respectively produced by rev and env genes. Due to lack of these genes, the virus cannot replicate.

Researchers have used HIV lacking rev and env genes for therapeutic purposes. T-Lymphocytes from HIV-infected patients are removed, and mutant viruses are inserted into them. The modified T-lymphocytes are cultivated and injected into the patients. Due to lack of essential genes, the viruses (HIV) cannot multiply, but they can stimulate the production of CD_8 (cluster determinant antigen 8) cells of T-lymphocytes. CD_8 cells are the killer lymphocytes. It is proved in the laboratory studies that these lymphocytes destroy the HIV-infected cells.

b. Genes of HIV proteins:

Some genes synthesizing HIV proteins are attached to DNA of mouse viruses. These genetically-modified viruses are injected to AIDS patients with clinical manifestations of the disease. It is believed that the HIV genes stimulate normal body cells to produce HIV proteins. The latter in turn stimulate the production of anti-HIV antibodies which prevent the HIV replication in AIDS patients.

c. Gene to inactivate gp120:

gp120 is a glycoprotein (molecular weight 120 kilo Daltons) present in the envelope of HIV. It is absolutely essential for binding of virus to the host cell and to bring replication. Researchers have synthesized a gene (called F105) to produce an antibody that can inactivate gp120.In the anti- AIDS therapy, HIV-infected cells are engineered to produce anti-HIV antibodies when injected into the organism. Studies conducted in experimental animals showed a drastic reduction in the synthesis of gp120 due to anti-AIDS therapy. The production of F1IV particles was also very reduced. There are some attempts to prevent AIDS by antisense therapy.

Advantages of Gene Therapy

Gene therapy can cure genetic diseases by addition of gene or by removal of gene or by replacing a mutated gene with corrected gene.

Gene therapy can be used for cancer treatment to kill the cancerous cells.

Gene expression can be controlled.

Therapeutic protein is continuously produced inside the body which also reduces the cost of treatment in long ter

The Future of Gene Therapy:

Theoretically, gene therapy is the permanent solution for genetic diseases. But it is not as simple as it appears since gene therapy has several inbuilt complexities. Gene therapy broadly involves isolation of a specific gene, making its copies, inserting them into target tissue cells to make the desired protein. The story does not end here.

It is absolutely essential to ensure that the gene is harmless to the patient and it is appropriately expressed (too much or too little will be no good). Another concern in gene therapy is the body's immune system which reacts to the foreign proteins produced by the new genes.The public, in general, have exaggerated expectations on gene therapy. The researchers, at least for

the present, are unable to satisfy them. As per the records, by 1999 about 1000 Americans had undergone clinical trials involving various gene therapies.

Unfortunately, the gene therapists are unable to categorically claim that gene therapy has permanently cured any one of these patients. Some people in the media (leading newspapers and magazines) have openly questioned whether it is worth to continue research on gene therapy. It may be true that as of now, gene therapy due to several limitations, has not progressed the way it should, despite intensive research. But a breakthrough may come anytime, and of course, this is only possible with persistent research. And a day may come (it might take some years) when almost every disease will have a gene therapy, as one of the treatment modalities. And gene therapy will revolutionize the practice of medicine.

Probable questions:

1. Define Gene therapy.
2. Describe different strategies of gene therapy ?
3. Define somatic cell gene therapy and germ cell gene therapy?
4. What is ex vivo gene therapy and in vivo gene therapy?
5. How retroviruses are used in gene therapy?
6. How gene therapy is used in treatment of Cystic fibrosis ?
7. What is suicide gene therapy?
8. Describe gene therapy treatments for AIDS?
9. What is are the advantages of gene therapy?
10. Write about the future of gene therapy?
11. What is Differentiation therapy? Explain.
12. How differentiation therapy an be applied in cancer treatment ?

Suggested Readings:

1. Principles of Genetics. Snustad and Simmons.
2. Genetics . Verma and Agarwal.
3. Principles of Genetics by Tamarin.
4. Biotechnology by V. Kumaresan

DISCLAIMER: This Self Learning Material (SLM) has been compiled from various authentic books, Journals articles, e-journals and other web sources.