CELL BIOLOGY AND MOLECUALR BIOLOGY

(DBOT23)

(MSC BOTONY)

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UNIT - I

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Lesson 1

ULTRA STRUCTURAL ORGANISATION OF PLANT CELL, CELL WALLS, PLASMA MEMBRANE

OBJECTIVES

1.1 ULTRA STRUCTURAL ORGANISATION OF PLANT CELL

i) Introduction

Cell Biology may be defined as that branch of science which deals with the morphology, physiology and biochemistry of the cell. This definition raises the question what is a cell? A cell is a fundamental unit of biological activity. It is the smallest portion of the organism that exhibits the

properties characteristic of living material like reproduction, mutation, metabolism and sensitivity. We can define a cell "as a unit of biological activity delimited by a selectively permeable membrane and capable of self reproduction in a medium free of other living systems". This type of definition is necessary to distinguish the cell from a virus which is also a self reproductive biological unit but does not have a fine selectively permeable membrane and is capable of reproduction only within a cell.

ii) Plant Cell Organisation

The cells of algae, fungi, protozoa, higher plants and animals are known as Eukaryotic cells. These cells differ from prokaryotes in having a distinct membrane bound nucleus and some membrane bound cytoplasmic structures called cell organelleles with some special functions. Again plant and animal cells differ in certain characterstics.

1) Plants have a distinct cellulosic cell wall outside the plasma membrane while in animals, cell wall is absent hence plasma membrane is the outer most boundary in the case of animals.

2) Plastids which are responsible for the synthesis of food material utilizing atmospheric CO_2 and sunlight by the process of photosynthesis (chloroplasts) are absent in animals cells while they are present in plants.

3) Further vacuoles are present in plants while these are absent in animals.

4) Centrioles which are helpful in determining the polar asters during cell division are present in animals while these are absent in almost all plant cells except certain motile cells which contain cilia and flagella. Hence mitosis in the case of plants is anastral while in animals it is always astral.

The cells despite their great variety of shapes and sizes share many common features. The form and function of the cell are inseparable and an organized activity is associated with an organized arrangement of parts. This is what is known as principle of complementarity. There is no typical plant or animal cell that contains all the features we wish to examine. However, we can think of a generalized cell. Our general plant cell has a rigid non-living cellulosic envelope formed by the protoplast called cell wall which serves the mechanical function of giving support. The outer most living boundary of the cell is known as plasma membrane which separates it from the environment and through which the substances entering or leaving the cell must pass. Our general cell contains a nucleus surrounded by a nuclear membrane with pores here and there. This is the centre of control of all cellular activities. With in the nucleus the hereditary material DNA is present in the form of chromosomes along with protein, the DNA and histones (basic proteins) are complexed into chromatin which is packed in the chromosomes. The nuclear pores are responsible for maintaining the nucleocytoplasmic relations. In the non-dividing nucleus one or two round structures are present called nucleoli (single nucleolus). The nucleolus is responsible for the synthesis of ribosomal RNA. The cytoplasm of the nucleus is called nucleoplasm. A cell without a

nucleus is a cell with out future since it can not give viable progeny. The remainder of the cell is the cytoplasm. Here there are various membrane systems like endoplasmic reticulam and golgi complex and particulate organelles like mitochondria, plastids, lysosomes, ribosomes, vacuoles, microtubules and filaments, secretary products like glycogen, mucous startch etc., and soluble components that through chemical reactions control the problems of synthesis, energy transfer and conversion necessary for the tasks that cells perform are present (Fig. 1.1). With the advent of electron microscope and other sophisticated technologies like freeze etching, freeze drying, X-ray diffraction, auto radiography, ultra centrifugation, spectroscopy, etc it has been possible to study the ultra structure and functions of various cell components in detail (see lessons 1, 3, 4 & 5).

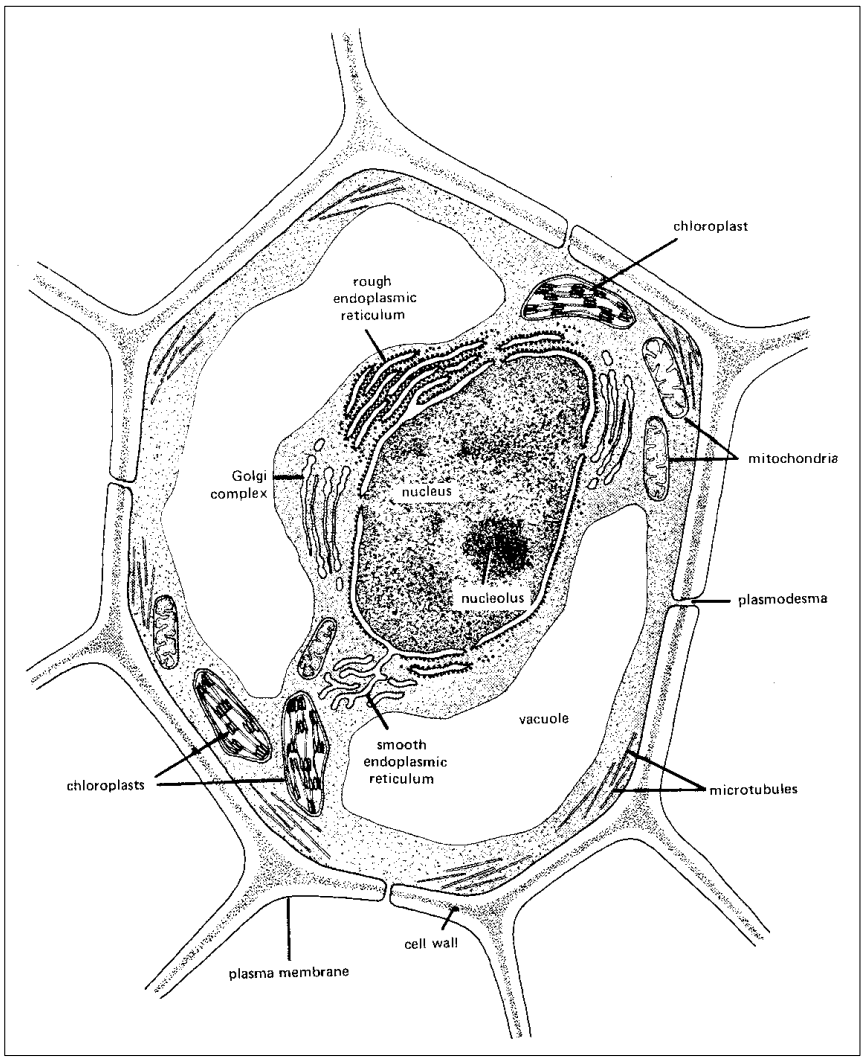


Fig 1.1 Ultra structural organisation of a plant cell

1.2 CELL WALL

i) Structure of cell wall

Plant cells are characteristically enclosed by a rigid non-living envelope formed by the protoplast called cell wall which serves the mechanical function of giving support. Further, it prevents the movement of water into the cell to the point where the protoplast would simply burst open. The tendency of water to enter the cell causes it to swell until pressure is exerted on the cell wall. It is this hydrostatic pressure that maintains the turgidity of the plant parts. Further, cell enlargement as a result of water moving into the cell is possible only as long as the cell wall remains sufficiently extensible.

The primary wall, the first layer to be laid down consists of a frame work of microfibrils made up of the polysaccharide cellulose embedded in a matrix of several other polysaccharides and glycoproteins. Between two adjacent primary walls is the middle lamella which consists mainly of another polysaccharide pectin and which acts as an intercellular cement binding cells together. While a cell is enlarging, the primary wall is thin, elastic and capable of great extension. Some thickening of wall can occur during elongation. In general, this happens after the cell has reached its maximum size. After this time, a secondary wall may be laid down between the primary wall and the plasma membrane. The secondary wall may be thick or thin and of varying degrees of hardness or colour. It is the part of the cell that give various woods and plant fibers, their particular character and form which is derived the cellulose used in the manufacture of rayon, nitrocellulose, cellophane and certain plastics (Fig. 1.2).

ii) Functions of cell wall

a) It gives mechanical strength and protection to the cell.

b) It is involved in free transport of movement of materials and metabolites in and out of the cell.

c) It counteracts physically the osmotic pressure produced by the cell contents.

d) It plays an important role in cell expansion.

e) Many enzymatic activities occur with in the cell wall. Hence, although cell wall is non-living it is a highly functional region outside the plasma membrane the cell.

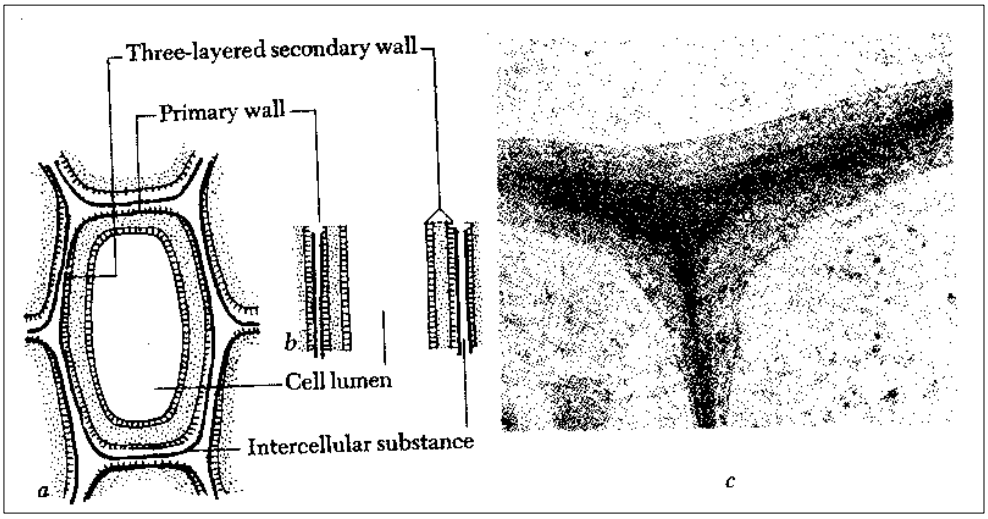


Fig 1.2 Typical wall structure of matured and lignified plant cells.

(a) Cross section, showing arrangement of the various layers and the complex structure of the secondary wall, (b) Longitudinal section through a similar cell. (c) Electron micrograph of the cell walls of three adjacent cells; the darker middle area is the middle lamella; the lighter portions, the primary wall.

1.3 PLASMA MEMBRANE

1) Introduction

Plasma membrane is the outermost living boundary of the cell which separates it from the environment and through which the materials entering or leaving the cell must pass. The existence of this selectively preamble membrane was inferred even before the advent of electron microscope. This inference was based on the fact that cells could swell and shrink and that when their surface was torn out by a dissecting needle the contents would ooze out studies with electron microscope confirmed the universal existence of this membrane.

2) Membrane structure

Plasma membrane is invisible in light microscope because it is 100 A^o or 0.01 μm thick and thus below the limits of the ordinary resolution. The permeability properties of the membrane gave first indication of the membrane structure. The permeability properties observed for plasma membrane are: (a) ionic or electrically charged compounds penetrate more slowly than non-ionic compounds, (b) Small molecules penetrate more rapidly than large molecules, (c) More soluble a compound in lipid, more readily it can enter a cell. These properties of plasma membrane suggest that the membrane is carrying a positive electrical charge that could impede the passage of positive ions, contains pores through which small molecules can pass and consists of atleast a part of a lipid or fatty film into and out of which the fat soluble compounds can move.

The plasma membrane is trilaminar and the term unit membrane can be applied to this structure because all the membranes have lipo-protein composition (Figs. 1.3, 1.4 and 1.5).

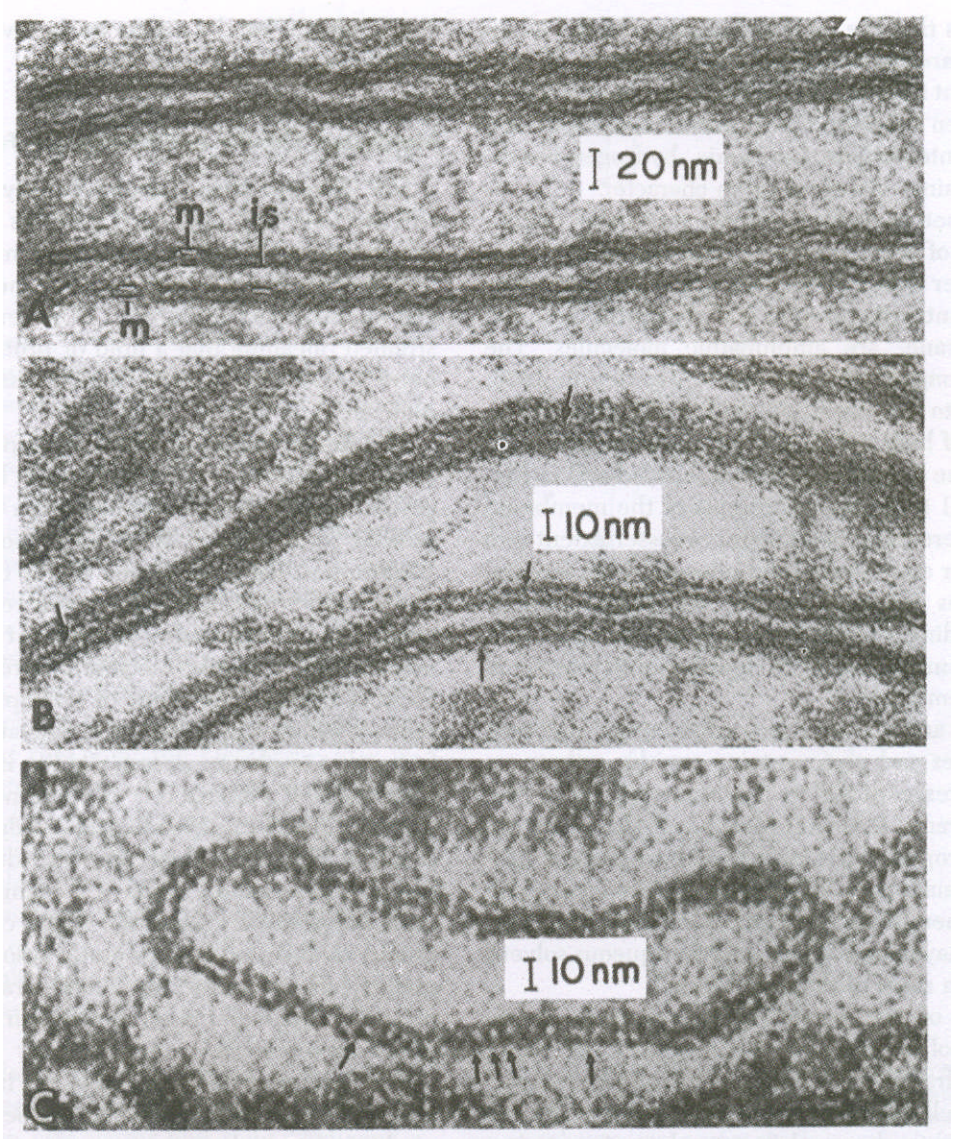


Fig 1.3 **A,** electron micrograph of cell membranes of intestinal cells (m), showing the three-layered structure (unit membrane), is, intercellular space. x 240,000. **B**, cell membranes in the rat hypothalamus showing the unit membrane structure and, with arrows, some finer details across the membrane. The upper arrows indicate a region in which the two cell membranes are adherent (tight junction) and the intercellular space has disappeared. x 360,000. **C**, the same as B, showing fine bridges (arrows) across the unit membrane. x 380,000. (From E. De Robertis, unpublished).

The protein constituent gives to the cell the structural integrity and flexibility. Since the protein molecules can fold and unfold, the cell membrane can also expand and contract and through molecular spacing control which molecules can enter the cell from outside environment or pass to the environment from inside of the cell. This sort of membrane is said to be **selectively permeable** and this will permit growth and movement either for the cell or for a localized region as in the case of Amoeba. Physiological, analytical and microscopical studies are consistent to the membrane structure that a double lipid layer is sand witched between two protein layers. When lipid is extracted from a known number of cells and spread as a monolayer on water, covered twice the area calculates for the surface area of the cells.

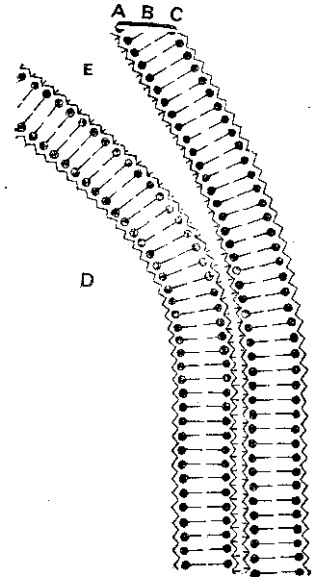


Fig 1.4 Schematic diagram of unit membrane, showing the molecular structure of unit membrane where (A) is the layer of proteins and carbohydrates on the outside of membrane. (B) is the lipid cone. (C) is the protein layer of the innerside (D) is schwann cell cytoplasm (E) Extracellular material.

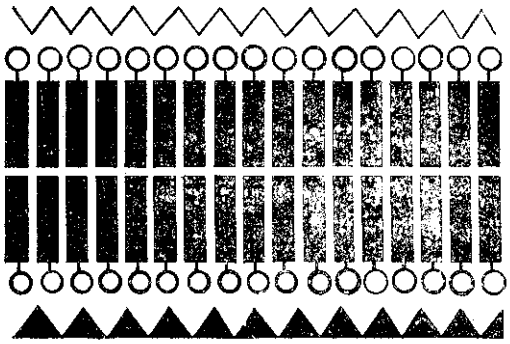


Fig 1.5 Schematic representation f unit membrane where circular polar groups are of lipids, non polar chains of bars are of carboons and monopolar zig zag lines are of non-lipids.

iii) Molecular Models of Plasma membrane

Previously membranes were thought to be static structures functioning only to separate the cells from the external environment. It has now been established that membranes are dynamic structures and are involved in most of the cellular activities. Thus the organization of plasma membrane of different cells and cellular organellels is therefore essential in understanding the mechanism of cell function. Membranes are composed of lipids, proteins and small amounts of carbohydrates. The chemical composition of the membrane is not constant for all cell types. There is considerable variation in the amount of proteins and lipids in different organisms. The ratio of protein to lipid varies from 80 : 20 in bacteria to 20 : 80 in some nerve cells. But in most of the membranes the ratio is 50 : 50. The lipid components of the membrane consists of phospholipids, glycolipids and steroids. Due to this diversity of membrane composition different models have been proposed to know the structure and organization of membrane.

a) Lipid Monolayer Model of Langmuir 1917

A first scientific attempt to know the structure of the membrane was made by Langmuir (1917) who suggested that the membrane was composed of phospholipids of one molecule thick. It was shown by an experiment in which the phospholipid was spread on water. This formed a layer of one molecule thick on water surface. Phospholipids are known to be amphipathic molecules which contains both hydrophilic and hydrophobic regions. Langmuir interpreted his model that the hydrophilic or head groups of the lipid molecules remain attached to the water surface and the hydrophobic tails remain free towards the air (Fig. 1.6).

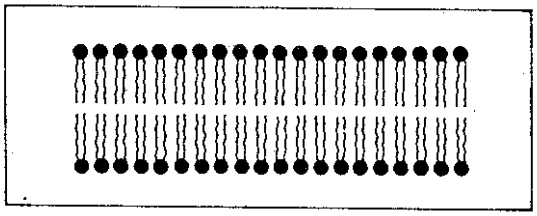


Fig. No. 1.6 Lipid bilayer model of Gorter and Grendel

c) The Danielli - Daveson Model 1934

The results of Harvey and Danielli showed that the surface tension of the cell membrane was higher than that of pure lipids. Hence, they concluded that biological membranes could not be of lipids alone. Later Danielli and Daveson proposed a molecular model of the membrane in which the hydrophilic head groups of the lipid molecule is covered on both sides by protein layer. The proteins are attached to the hydrophilic head groups of lipid bilayer by ionic bonds. They also postulated the existence of pores across the membrane (Fig. 1.7).

With the advent of electron microscope, the invisible structure of plasma membrane was noted. With osmium tetroxide as the only fixative membrane structure appeared only as a single line on the cell surface. Later Robertson used (1964, 66) permanganate as a fixative and showed the trilaminar structure of the biological membrane.

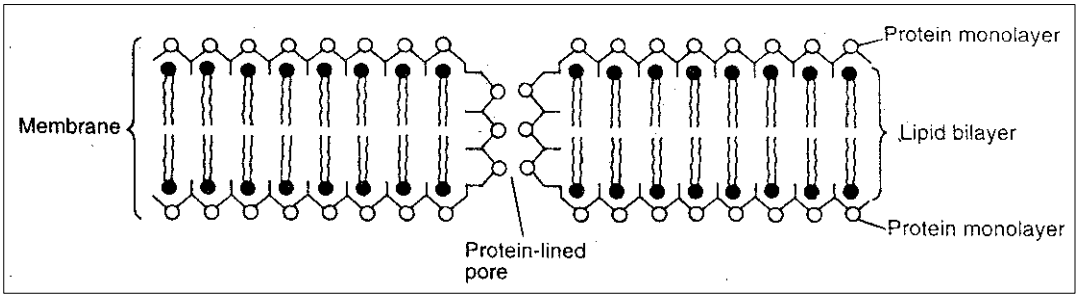


Fig No. 1.7 Danielli-Davson Model

d) Robertson's Model (1964, 66) or Unit Membrane Model

With the permanganate fixative, electron micrographs of fixed membranes revealed a general structure for all cell membranes. The unit membrane model suggested that all cell membrane are trilaminar and are composed of phospholipids and proteins. A layer of carbohydrates and proteins are on the outer membrane. Next comes the lipid layer which is double in nature. The lipid molecules are made up of polar hydrophilic and non-polar hydrophobic ends. Although the original model of Robertson does not indicate any chemical asymmetry recent model indicates a chemical asymmetry in the membrane surface. In the outer layer there is a higher concentration of muco polysaccharides or muco protein with a dominance of protein on the inside (Figs. 1.4 & 1.5).

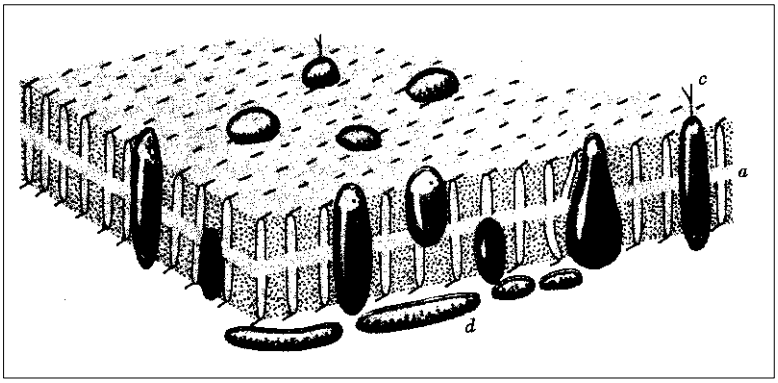
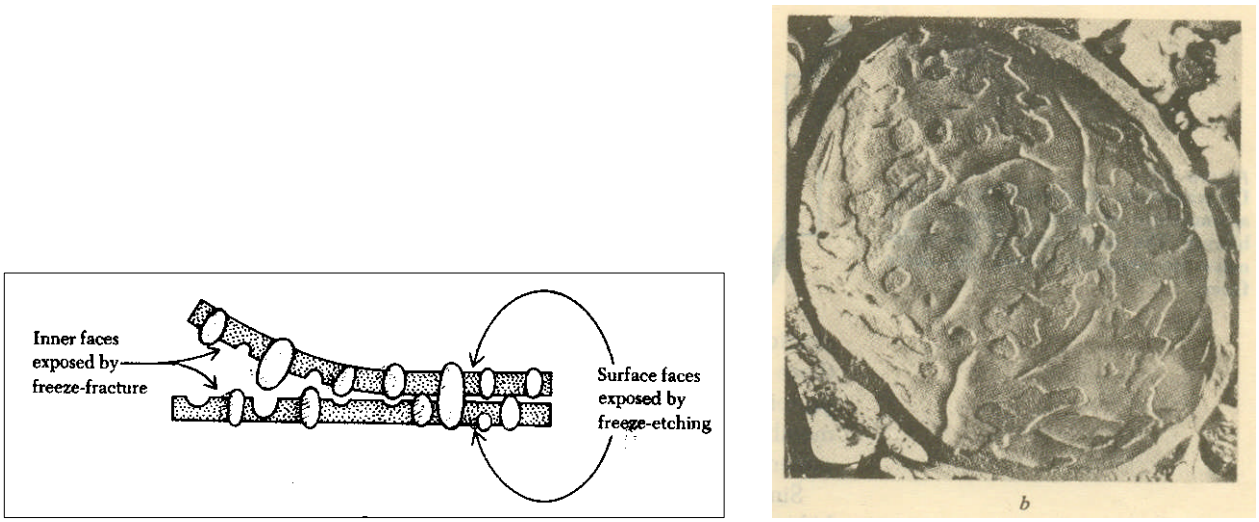


Fig. 1.8 The cell boundary

e) Fluid Mosaic Model

Although this unit membrane concept may be partially correct it is now clear that membrane structure is neither as simple nor as uniform as was formerly believed. Many membranes have been shown to display a particulate appearance in the electron microscopy (Fig 1.9a & b). This is

especially striking following preparation of membranes by freeze fracturing and freeze etching. These particles are thought to be globular proteins embedded in at the surface of the lipid matrix of the membrane. The proteins present at the surface are the extrinsic ones and embedded proteins are called intrinsic proteins. Some of the proteins confined to the inner surface such as spectrin determine the distribution of other protein components. The membranes differ in the amounts of proteins and lipids and also in chemical constitution thus providing scope for the wide range of physiological activities displayed by different membranes. The control of transport, cell to cell recognition immunological response and even cell movement must themselves depend on which enzyme systems comprise the protein complements of the membrane and how these proteins are arranged in relation to each other and to the various lipids that are present. The membranes are not static. There is a considerable freedom of movement within the membrane of its constituent molecules.



(a) (b)

Fig 1.9 (a) Diagrammatic representation of how fracture faces and surface faces of membranes are exposed by freeze-fracturing and freeze-etching, (b) Electron micrograph of faces of yeast plasma membrane exposed by freeze-fracture and freeze-etching. Note the arrays of particles in the membrane (Courtesy of Dr. S.C.Holt).

The most favoured model that can explain all the above mentioned properties of the membranes is the so called fluid mosaic model put forward by S.J. Singer and G.L. Nicolson (1972). According to this model there is a rather continues lipid bilayer into which the integral proteins of the membrane are intercalated. Both these components (lipids and proteins) are capable of translational diffusion within the over all bilayer. The mosaic model of the membrane is supported by the results of freeze etching techniques in which protein particles are shown at the plane of cleavage of bilayer.

The Mosaic arrangement implies that (i) the macro molecules have a characteristic asymmetry (ii) that they are oriented for carrying information across the bilayer and (iii) that they have considerable freedom of movement within the bilayer (fluidity). The fluidity of the lipids depends on the temperature and degree of saturation. With freeze fracturing it is possible to demonstrate the movement of protein particles in different experimental conditions. The fluidity of lipids is supported by many indirect studies based on X-ray diffraction, different thermal analysis and electron spin techniques. The fluidity of integral proteins is supported by experiments on cell fusion.

iv) Molecular Organization of Red Blood Cell Membrane

The first step in the study of molecular organization of the membrane consists of isolation of the cell membrane. The easiest to isolate is the red cell membrane obtained after hemolysis (The cells are treated with hypotonic solutions) that produce swelling and loss of the hemoglobin content (e.g. hemolysis). The red cell ghost contains 52% protein 40% lipid and 8% carbohydrate that is bound to lipid (glycolipids) and proteins (glycoproteins). The lipids form a rather continuous bilayer in which the outer layer contains mainly lecithin and spingomyelin and the inner one phosphatidyl ethanolamine and phosphitidyl serine (lipid asymmetry). The oligo saccharides present in glycolipids and glyco proteins are located exclusively on the external surface (Fig. 1.10).

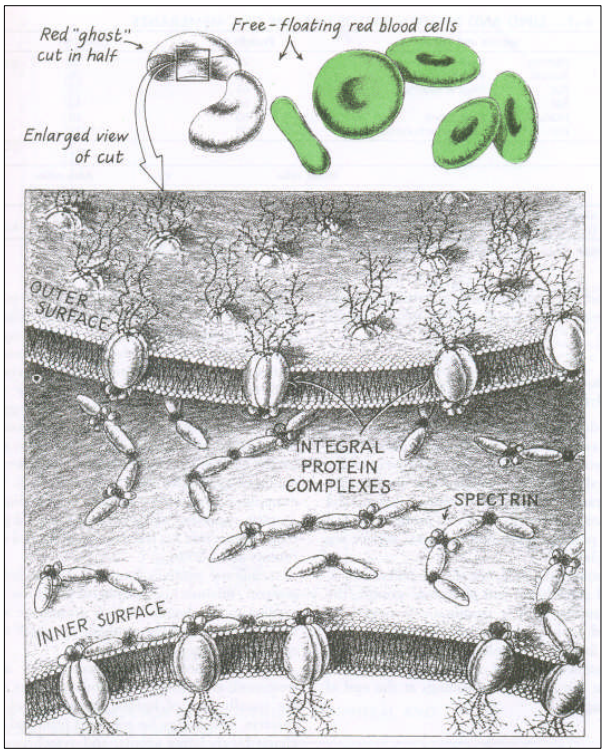


Fig 1.13 Diagram showing red blood cells, a red cell ghost cut in half, and a view of the molecular organization of the membrane according to the "fluid mosaic" model. Integral protein complexes are represented with the oligosaccharide chains sticking out on the outer surface. At the inner surface the peripheral protein is represented. (Courtesy of G.L. Nicolson, 1978).

Proteins play various roles, mechanical, transport, receptor, antigenic and enzymatic. They are classified by the degree of their association to the membrane. Proteins are peripheral (extrinsic) or integral (intrinsic). The various proteins may be isolated by polyacryl amide gel electrophoresis (PAGE). Every protein in the red cell membrane is asymmetrically distributed. The peripheral proteins spectrin and actin form microfilaments associated with the inner surface of the membrane. Other peripheral proteins are glyceraldehyde 3p-dehydrogenase (G_3PD) and the protein of band 4.2.

The major intrinsic protein of band 3 is 93000 daltons, spans the membrane and is present as a dimer. The major glycoprotein is glycophorin with a molecular weight of 55000 daltons which also spans the membrane and has several antigenic sites. There are some 30 enzymes in the cell membrane and these have an asymmetrical distribution.

1.4 SUMMARY

Ultrastructural study of the plant cell revealed a cellulosic cell wall, the outermost living boundary plasma membrane, endomembranes and cell organelleles such as nucleus with chromosomes and nucleoli plastids, mitochondria, lysosomes, ribosomes, vacuoles microfilaments and microtubules etc. The animal cell differs from the plant cell in the absence of cellulosic cell wall plastids and vacuoles.

The cell wall is cellulosic and is made up of non-living cells. It consists of primary wall, secondary wall and the intercellular middle lamella. It gives mechanical strength and protection to the cell.

The plasma membrane is the outermost living boundary of the cells and is selectively permeable. It is of unit construction and is made up of phospholipids and proteins. There are several molecular models proposed to explain the structure of the plasma membrane they being, lipid monolayer model (Langmuir, 1917), Lipid bilayer model of Gorter and Grendel (1926). The Danielli – Daneson Model (1934), Unit membrane model by Robertson (1964, 66) and fluid mosaic model (Singer and Nicolson, 1972). Of all the models, the fluid mosaic model explains all the properties of the membrane. According to this model, there a rather continuous lipid bilayer into which the integral proteins are intercalated. The macromolecules have a characteristic asymmetry and they have freedom of considerable movement within the bilayer. As a typical membrane, the molecular organization of red cell membrane with extrinsic and intrinsic proteins is described.

1.5 MODEL QUESTIONS

1. Describe the ultrastructure of a plant cell.
2. Give an account of the structure and various molecular models suggested for plasma membrane.

3. Write short notes on:
   a) Cell wall
   b) Fluid mosaic model
   c) Red cell membrane structure.

1.6 REFERENCE BOOKS

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UNIT - I

Paper-VII : CELL BIOLOGY AND MOLECUL

Lesson 2

PLASMA MEMBRANE

OBJECTIVES

2.1 ARTIFICIAL MODEL SYSTEMS - LIPOSOMES

The lipid bilayer structure of cell membranes has been further confirmed by comparing the natural membrane with artificial membranes. These artificial lipid layers can be made either by placing a drop of lipids in a small hole separating two compartments containing water and by vibrating the suspension of lipid and water ultrasonically. Lipid monolayers and bilayers as well as myeline figures or crystalline phospho-lipid water phases with hexagonal configuration may be produced and analysed with polarisation microscopy, X-ray diffraction or electron microscopy (Figs. 2.1 & 2.2). Such studies may give information about the relative position of polar and non-polar groups and their association with proteins. Some inferences based on studies with the electron microscope may be made about the electron density images.

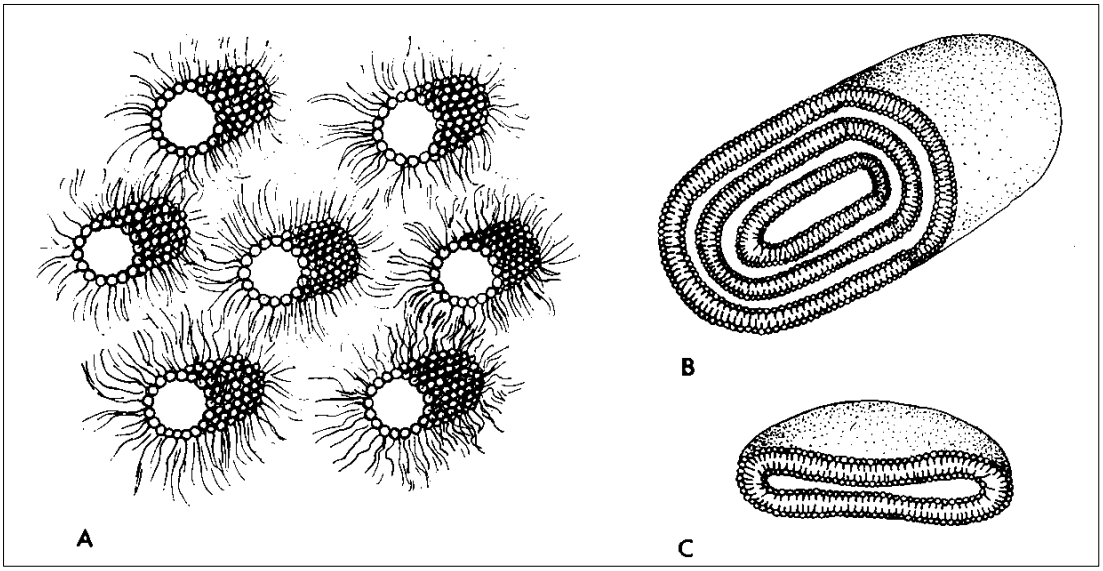

Fig No. 2.1 Different types of phospholipid-water systems. **A,** hexagonal phase in a partially hydrated phospholipid. Observe that the water is contained within the cylinders, which are limited by polar groups; **B,** multilayered smetic mesophase corresponding to one liposome. Water is contained between lipid bilayers; **C,** phospholipid vesicle, single bilayered structure of 2 nm (Courtesy of A.D. Bangham and D.A. Haydon, From Bangham, A.D., and Haydon, D.A.: Br. Med. Bull. 24: 124, 1968).

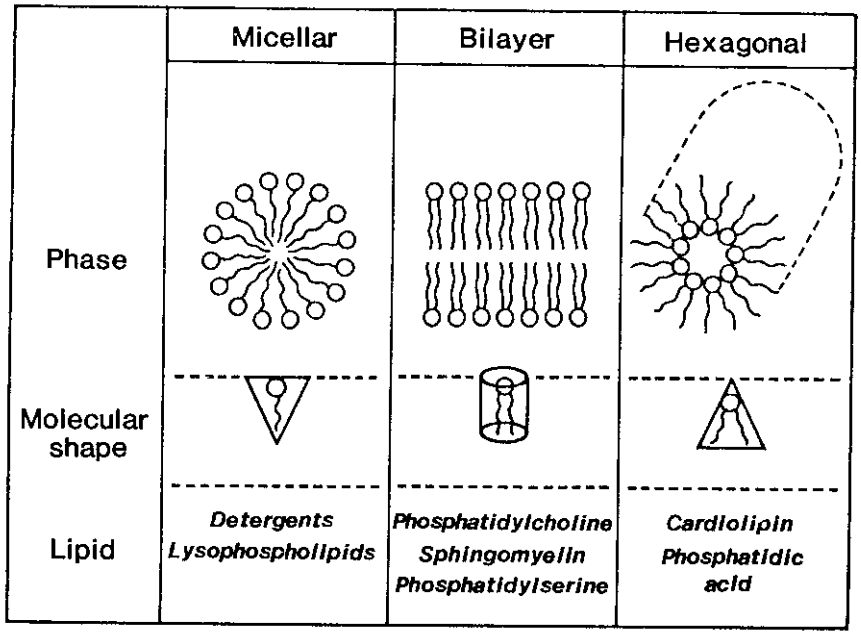

Fig 2.2 Diagram showing how the molecular shape of thelipids and the polar and nonpolar ends influences the type of phase produce, i.e., micellar, bilayer, and hexagonal.

Some special model systems of lamellar type are provided by multilayered liposomes and single bilayered phospholipid vesicles. These systems may have important applications in biology and medicine.

i) Liposomes

In 1965 Bangham defined a liposome as a special type of lamellar phase is which the water is self contained and the lipid molecules are disposed in bimolecular layers attached by their non-polar interfaces (Fig 2.1).The type of micellar, Bilayer Hexagonal lipists are shown in Fig. 2.2

ii) Applications

Liposomes constitute excellent vehicles for carrying different molecules which are protected within the lipid membrane into cells and tissues. Hence they have immense application in the field of medicine. Some examples are given below.

a) A missing enzyme can be delivered into an organism that has a heriditary deficiency of that enzyme.
b) Further more drugs for cancer (Chemotherapy) may be delivered more easily to the sites of attack.
c) Insulin may be given orally with liposomes, and
d) Specific antibodies can be delivered to certain cell types.

2.2 PINOCYTOSIS AND PHAGOCYTOSIS

It is of interest to note that free cells such as those in tissue culture can take in materials from the liquid environment by one or both of additional processes pinocytosis and phagocytosis. The former name is derived from the Greek words (pino = drink, cyto = cell) and the process is literally a drinking phenomenon. The flexible plasma membrane forms a channel to get liquids into the cell and then pinches off pockets that are incorporated into the cytoplasm (Fig. 2.3).

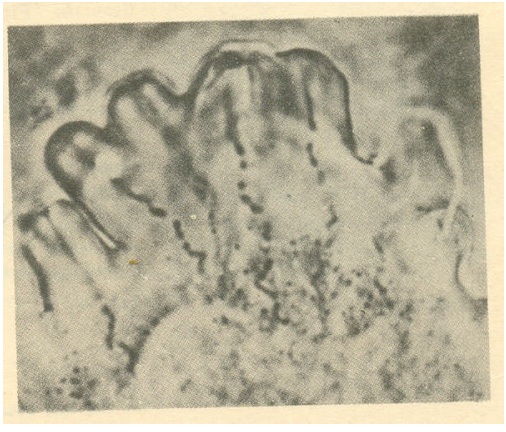

Fig 2.3 Photograph of the edge of a living amoeba, showing the pinocytotic channels (the dark lines converging toward the center of the cell). Liquids flow into the cell through these channels, to be pinched off as membrane-enclosed droplets; these eventually dissolve in the interior of the cell (Coursey of Dr. David Prescott).

By this device large molecules and various ions which are incapable of passing through the membrane can be taken up by cells. In phagocytosis arms of cytoplasm engulf droplets of liquid containing solid material such as bacteria and draw these materials into the cytoplasm where the digestive enzymes break the engulfed materials down into usable fragments (Fig. 2.4).

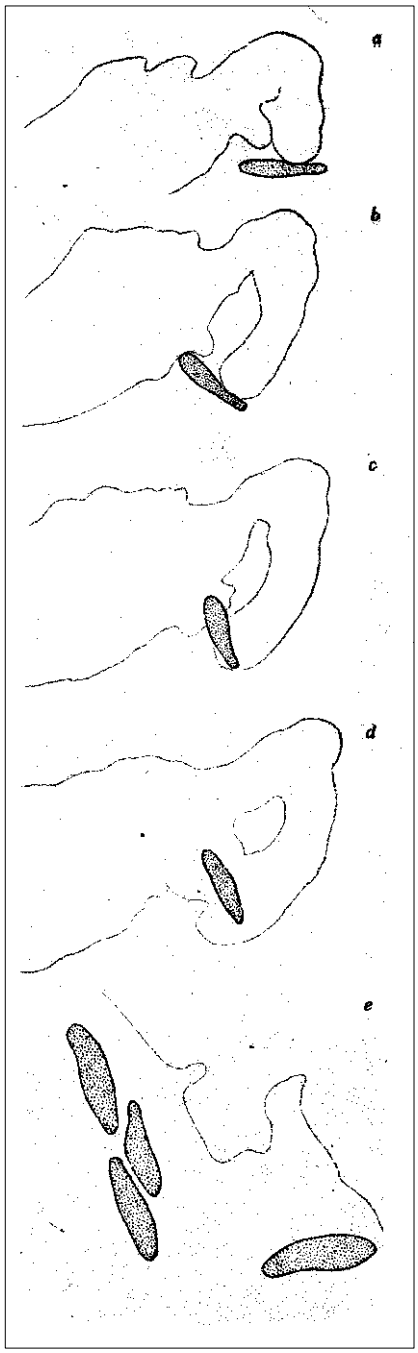

Fig 2.4 Phagocytosis as observed in an amoeba. The arm of cytoplasm, coming in contact with a paramecium, surrounds it and then draws it into the cytoplasm where it can be digested. (a-d). The process of enveloping. (e) A portion of the amoeba containing several phagocytized paramecia. →

We can therefore consider plasma membrane as a portion of the living cell. This point of view is strengthened by the fact that it has intimate connections with other internal membrane systems, its capability of limited repair if torn or punctured by a needle and its activity in a cell exhibiting pinocytosis, phagocytosis or mobility.

2.3 MODIFICATIONS OF PLASMA MEMBRANE

Plasma membrane is elastic, changeable and pliable in some cells, quite rigid and unyielding in others. It is smooth in Amoeba and ciliated in **paramecium**. This structure is not a simple envelop comparable to a thin plastic bag surrounding the cell contents. It is capable of undergoing several modifications.

i) Brush boarder and desmosomes

In some cases like columnar epithetical cells lining the small intestine of mouse this membrane is greatly convoluted and contain regions that show intimate connections - desmosomes with adjacent cells, while the upper surface forms a brush boarder in which the many projections microvilli provides a tremendous absorption area (Fig. 2.5 and 2.6).

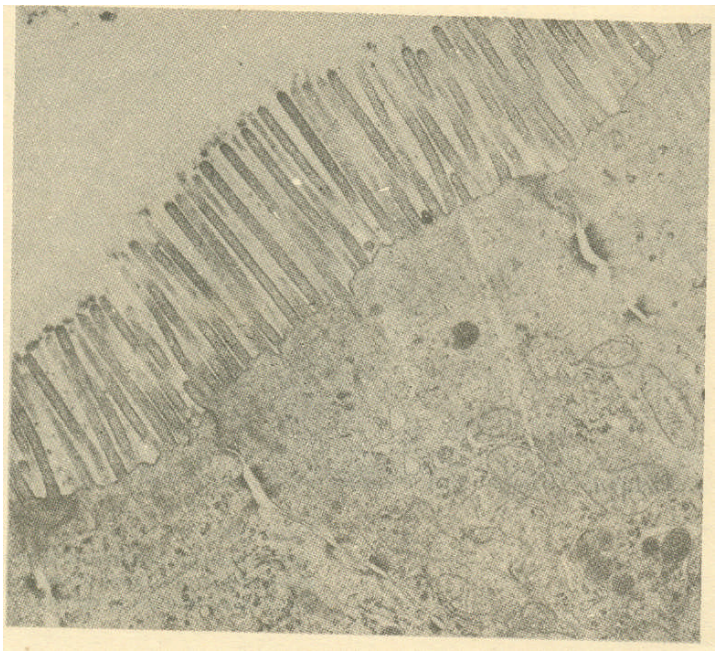

Fig. 2.5 Microvilli projecting from the surface of a columnar epithelial cell.
The glycocarlyx is apparent at the tips of the microvilli (Courtesy of W. McDaniel).

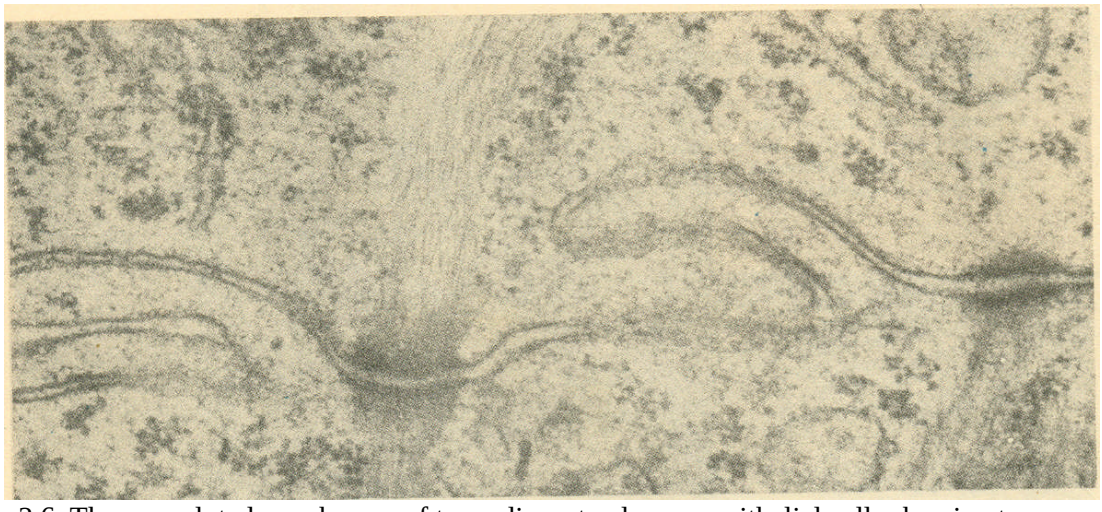

Fig. 2.6 The convoluted membranes of two adjacent columnar epithelial cells showing two prominent desmosomes. Microfilaments extend from the desmosomes into the cytoplasm. (Courtesy of Dr. Philpott).

ii) Intercellular attachments

Essentially four types of differentiations are present at the lateral surface of the epithelial cells (i) The macula adherence or desmosomes, (ii) The zonula adherence also called intermediary

junction or terminal bar, (iii) The zonula occuldense or tight junction, (iv) the gap junction or nexus (Fig. 2.7).

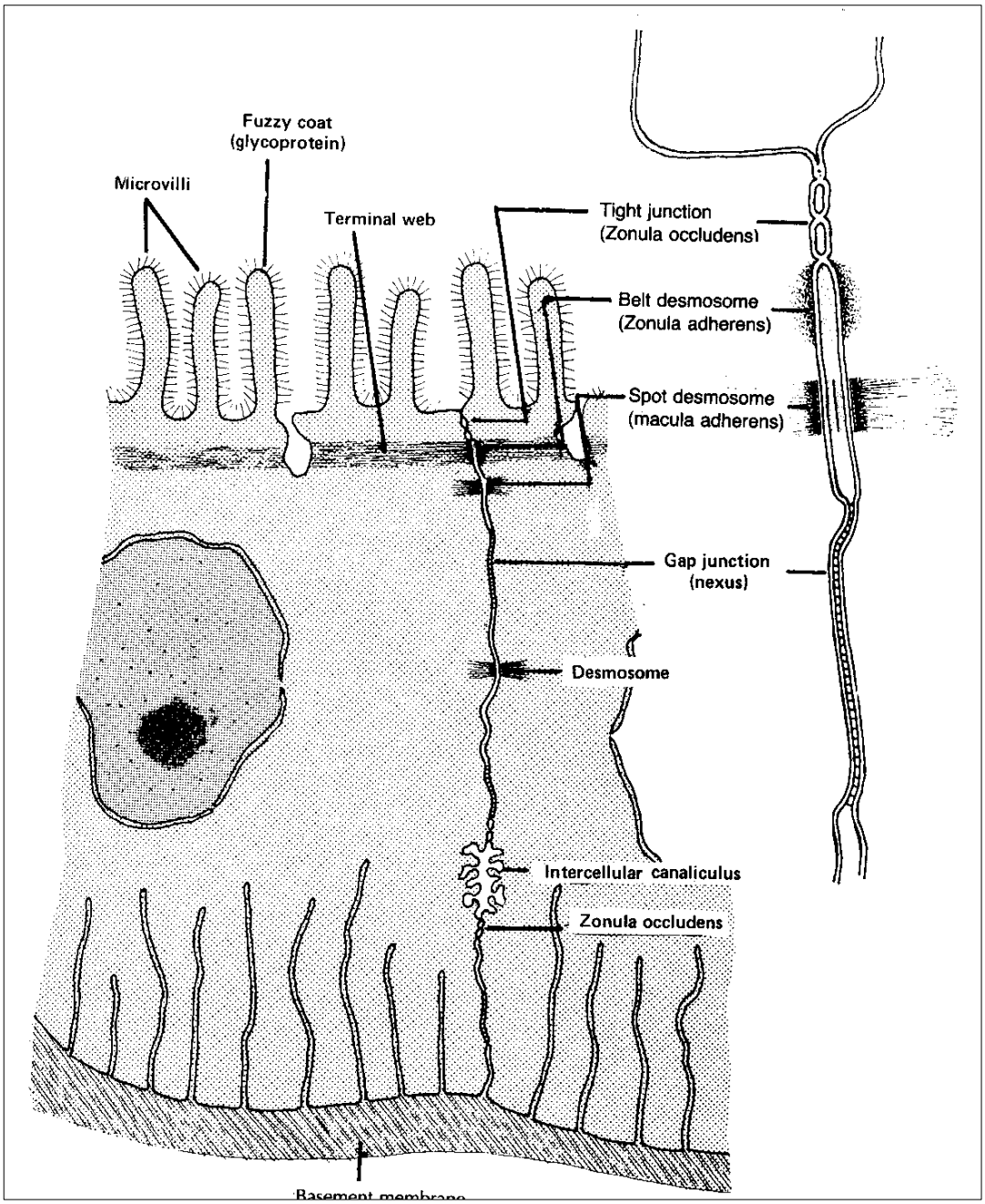

Fig 2.7 Diagram of an idealized columnar epithelial cell showing the main differentiations of the cell membrane. To the right, at a higher magnification, the series of differentiations found between two epithelial cells are indicated.

a) Desmosomes or Macula adherence

These are zones of attachment between two epithelial cells and have a mechanical function.

b) Zonula adherence or intermediary junction

These are attachments between the cells and also has a mechanical function. This is a less common type and encircles the cell like a belt. Tonofilaments are often absent.

c) Tight junctions or Zonula occludens

These are the regions in which two adjacent membranes fuse forming a kind of seal. At these points, the intercellular space disappears.

d) Gap junctions or nexus

These are essential in intercellular communications. They represent regions in which there are junctional channels through which ions and molecules can pass from one cell to another. Cells having gap junctions are electrically coupled i.e., there is a free flow of electrical current carried by ions. At the regions of gap junction the membranes are separated by a space of only 2-4 nm and there is a hexagonal array of 8-9nm particles in the centre of each particle there is a channel 1.5-2nm in diameter.

c) Myelin Sheath formation

In the case of a nerve cell or schwan cell or satellite cell the entire plasma membrane of one cell forms an elaborate structural system around a portion of another cell. The nerve fiber or axon is wrapped by schwan cell in a spiral fashion. Finally the cytoplasm is squeezed to the outside leaving axon surrounded by a multi-layered membrane system and is isolated from its surroundings. This sheath is called the myelin sheath which is thought to assist in the transmission of nerve impulses (Fig. 2.8).

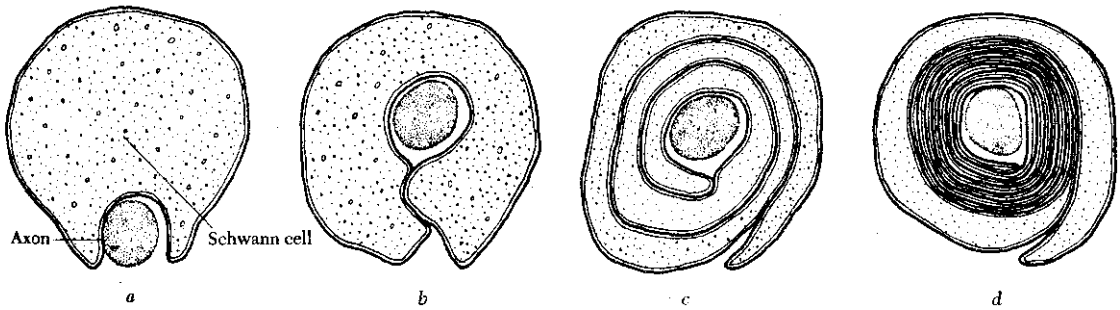

Fig 2.8 Schematic representation of the progressive envelopment of an axon by the membranes of the Schwann cell, as described by Dr. Betty B. Geren. Such an axon is said to be myelinated.

f) Modification in light receptor cells

Another modification of cell membrane can be seen in the light receptor cells rods and cones of the vertebrate eye. The outer segment of these cells is made up of a series of flattened discs which are about 500-1000 in some cells, staked one upon the other like coins. The discs are made from foldings of the cell membrane and appear as free floating structures and they represent light receptor surfaces of the eye (Fig. 2.9).

2.4 FUNCTIONS OF THE PLASMA MEMBRANE

i. Gives identity to the cell
ii. Protects cell organelles
iii. Helps in digestion through pino and phagocytosis
iv. Being permeable to water, water can pass through it (active and passive transport).
v. Helps in the transport of ions.
vi. Helps in the formation of cytoplasmic organelles.
vii. Regulates the out flow of exeretory material and water from the cell.
viii. Maintains the balance between the osmatic pressure of the inter cellular fluid and that of the interstitial fluid.

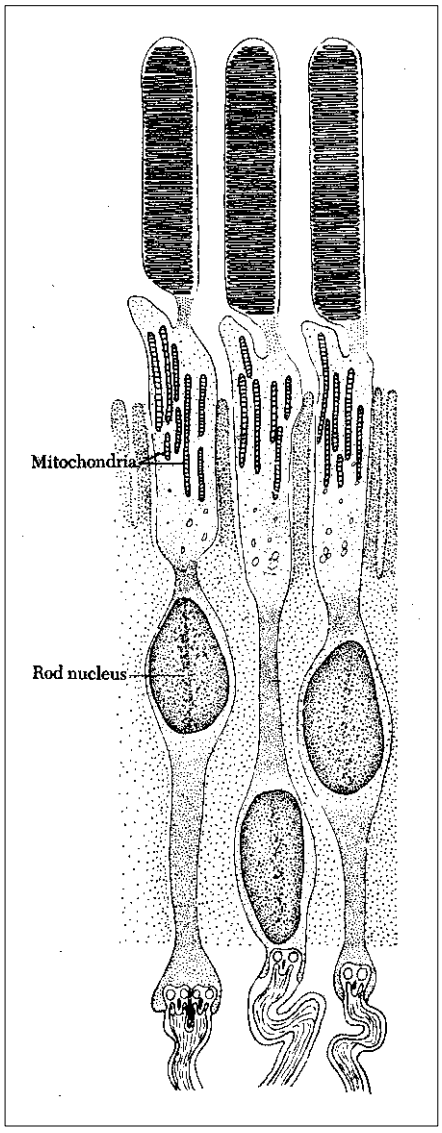

Fig. 2.9 Schematic representation of a rod (light-receptor) cell in the retina of the guinea pig. The discs at the top of the cell are folded and refolded to provide many layers of membranes, each of which contains light-sensitive pigments on its surface. The mitochondria are concentrated just below the light-sensitive area; the rod nucleus is also identified. At the base, each cell has an intimate connection with a nerve fiber.

2.5 MEMBRANE FLUIDITY AND MEMBRANE FUSION

The concept of membrane fluidity refers to the fact that both lipids and proteins have considerable freedom of lateral movement within the bilayer. Vectorial movement across the membrane is however is severely constrained (meaning that a lipid or protein in the outer half of the bilayer can not pass into the inner half).

Membrane fluidity is essentially a property of the lipids. Normally these are fluid at the body temperature and the main consideration is the degree of saturation of the hydrocarbon chains. Unsaturated fatty acids i.e. those containing double or triple bonds will have a lower melting point than saturated ones. In hybernating animals during the phase of hybernation there is a change in fatty acids that increases fluidity. Rapid changes in fluidity can be produced by methylation of phosphitadylethanolamine by methyl transferases present in the membrane, these in turn are regulated by receptors.

The fluidity of the membrane can be studied with a series of techniques that can be classified as physical or biological. The physical techniques are of two types (a) Those that involve minimal perturbation of the membrane such as X-ray diffraction and nuclear magnetic resonance (NMR) spectroscopy and (b) those that could use certain added molecules to monitor the specific sites of the membrane. Into this second class fall fluorescent microscopy which used fluorescent probes and electron spin resonance (ESR) spectroscopy which uses paramagnetic probes (e.g.:- nitroxide containing amphipathic molecules) that are introduced into the lipid bilayer. The data obtained by these techniques gives information on lipid protein packing, lateral diffusion of lipids, lipid-protein interactions, the fluidity of the membrane and the rate of transmembrane rotation of molecules across the bilayer.

The biological techniques involve light and fluorescent microscopy including freeze fracturing and radio-isotope labelling methods.

One of the simplest methods consists of binding of gold or carbon particles to the cell surface and observing under the light microscope. The movement of these particles can be seen on the surface.

In another experiment ligands such as antibodies and plant lectins are introduced which interact with cell surface receptors. If the ligands are labelled with fluorescent dyes their movement can be followed through fluorescent microscopy. E.g.:- capping phenomena. In this experiment the lymphocytes are treated with fluorescent antibodies to certain membrane antigens, it is possible to observe the capping phenomena. The antigens visibly displace the membrane forming patches. These patches move and aggregate at one pole of the cell producing a cap which is highly fluorescent. At this point the membrane may invaginate into vesicles which are internalized into the cytoplasm. The capping process can be inhibited by lowering the temperature so that the lipid bilayer solidifies (Fig. 2.10).

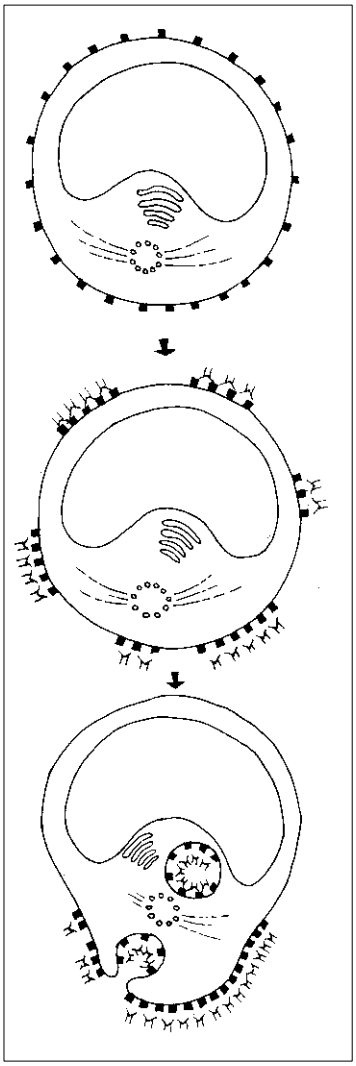

Fig 2.10 Diagram representing the phenomenon of "capping" in lymphocytes. Top, corresponds to the normal distribution of antigens on the cell surface. Middle, the antigens are now clustered into patches after being cross-linked with a bivalent antibody. Bottom, a cap is formed by the active transport of the patches toward the pole that contains the centrosomal-Golgi area. Observe that the membrane is being internalized by endocytosis. Antibodies are in red.

A classic experiment to prove fluidity and fusion of membranes is that of Frye and Edidin in which two different cultured cells having different surface antigens are fused. Cell fusion is achieved by the use of inactivated para influenza virus called sendai virus (named after a city in Japan). Sendai virus facilitates fusion of cells. There is fusion of two plasma membranes and cytoplasm producing a heterokaryon with two nuclei. The two cells are labelled with fluorescent antibodies of different colours such as fluorescent (green) and rhodamine (red) hence it is possible at the onset of fusion to recognize the parts of plasma membrane corresponding to each cell. However, intermixing occurs as the antigens are dispersed and the two colours become less and less detectable. After 40 minutes the intermixing is complete and the two antigens can no longer be distinguished. Again in this case also temperatures below the normal body temperature impair the intermixing by causing solidification of the lipid bilayer.

Fig. 2.11 represents the possible molecular mechanism of this fusion. When the two membranes approach each other and are under the influence of a fusogen (Sendai virus here) the following changes take place.

a) Displacement of intrinsic proteins to produce protein free zones and contact between the two lipid bilayers.

b) Formation of a hexagonal phase in between the two monolayers.

c) Complete fusion and reorganisation of the plasma membrane. The transition from bilayer to hexagonal phase seems to be essential in cell fusion as well as in other transitional processes such as endocytosis and exocytosis, blebbing of the plasma membrane and membrane biogenesis. The cell fusion experiments fully support the fluid mosaic model as there is clear evidence for the translational diffusion of lipids and protein with in the bilayer.

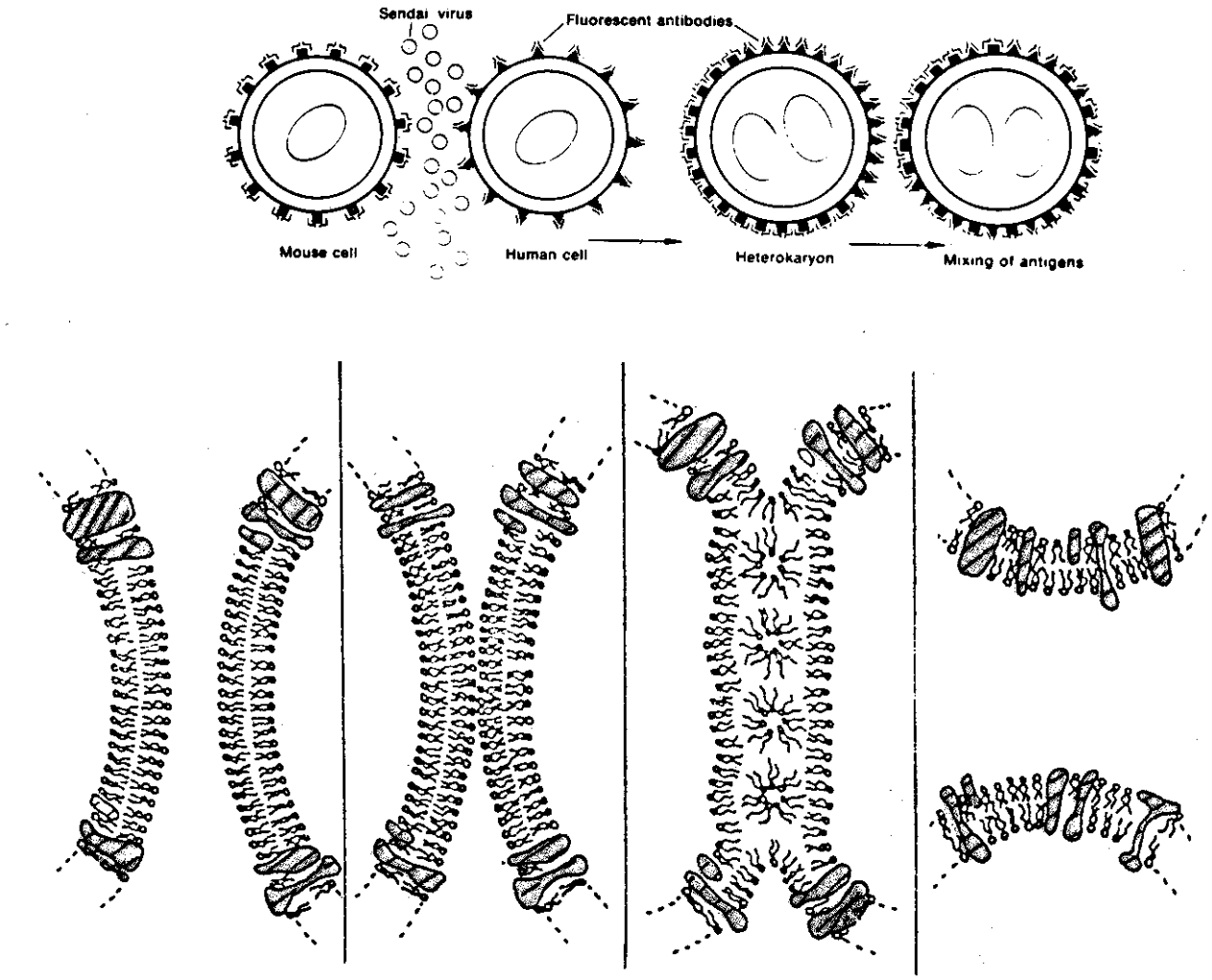

Fig. 2.11 **Top,** diagram representing the experiment of Frye and Edidin on cell fusion. A mouse and a human cell having different surface antigens labeled with fluorescent antibodies are fused by Sendai virus. The heterokaryon has each half of its surface covered with different types of antigens. After 40 minutes, mixing of the antigens occurs because of their movement in the plane of the membrane. **Bottom,** a possible interpretation of the molecular mechanism involved in cell fusion.

2.6 MECHANISMS OF TRANSPORT ACROSS THE MEMBRANE

The main role of plasma membrane is to regulate exchange of materials between the cell and its environment. Now we will consider the mechanisms involved in the transport of matter through the plasma membrane as well as the forces responsible for such movement. They can be dealt under two heads passive and active transport.

i) Passive transport: In passive transport no energy is expended. The first type of passive transport is passage through pores. In passive diffusion there is net movement of substances down a concentration gradient. Despite the presence of plasma membrane impeding this movement, many substances do diffuse rapidly across the membrane. As we have already seen water and other small molecules cross the barrier readily suggesting the presence of pores in the membrane. Although, pores have not been seen directly in the electron microscope it has been calculated that they must be very small and can occupy only a small portion of the surface area of the membrane. Further, such pores are not the permanent structural entities but represent transient and localized rearrangements in the molecular arrangement of a dynamic membrane.

Another mechanism that can account for rapid movement of solutes to which membranes are relatively impermeable is facilitated diffusion. The kinetics of entry of various sugars, amino acids and nucleosides strongly suggest that enzymes are involved in the acceleration of rate of diffusion through the membrane. Such enzyme systems presumably constitute part of the membrane it self and may act as carriers of the penetrating molecules. It should be pointed out that facilitated diffusion does not imply movement against a gradient and therefore is not directly dependent on expenditure of energy (Figs. 2.12 and 2.13).

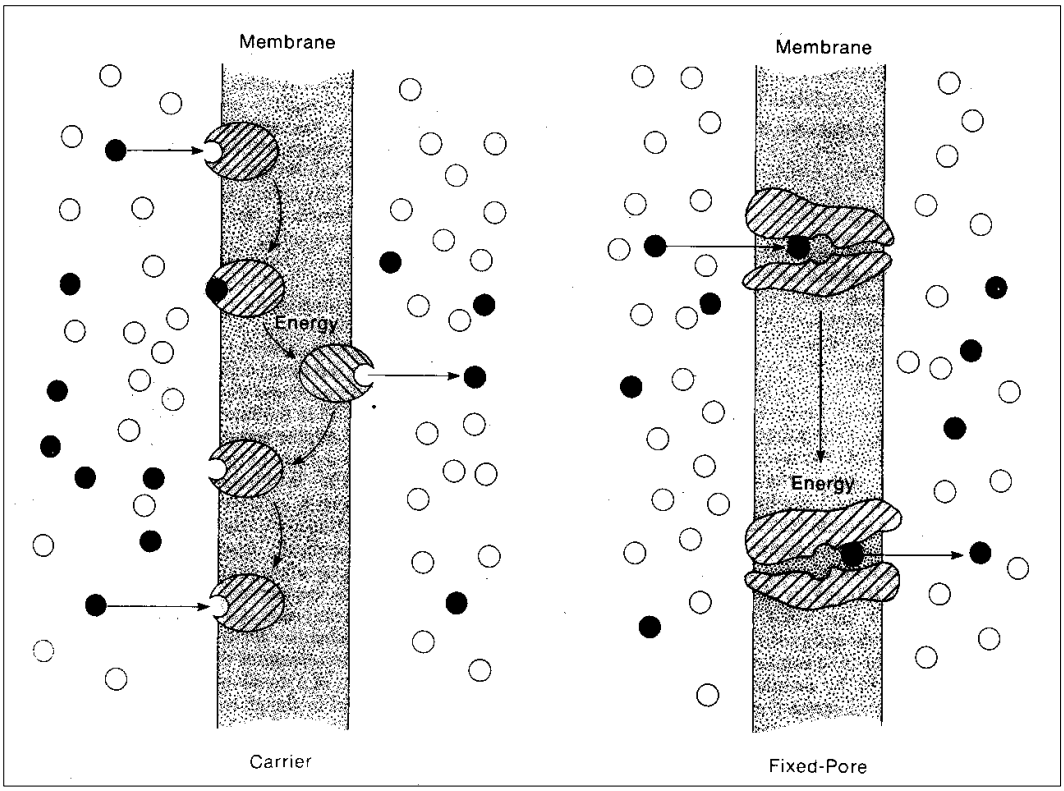

Fig. 2.12 Diagram representing the carrier and the fixed-pore mechanisms of selective transport

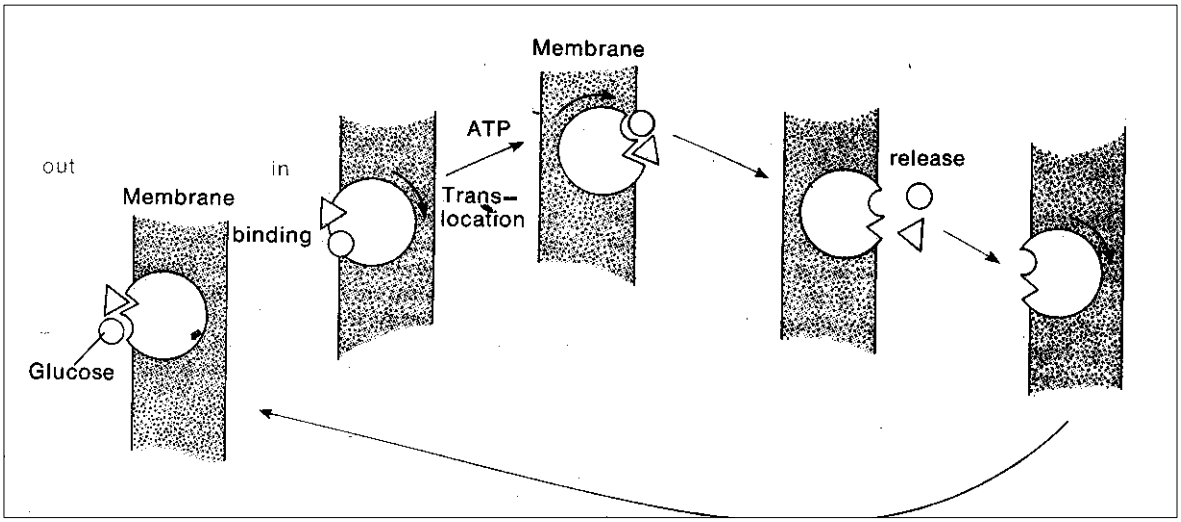

Fig. 2.13 Coupling of the transport of sodium and glucose by a hypothetical carrier mechanism.

ii) Active transport: In active transport mechanism there will be movement especially of ions across the membrane. Membranes maintain not only differences in concentration of substances between the inside and outside of the cell but also differences in electrical potential. Active transport therefore requires that energy be expended to do the work involved in uphill movement that is against an electrochemical gradient. The reactions involved in active transport must be coupled to reactions at the membrane in which free energy is made available. Enzymes capable of catalysing such exergonic reactions which provide the energy necessary to drive the pumping of compounds into and out of cell are believed to be integral components of the membrane.

Let us consider a case of active transport of (Na^+) across the membranes. For most concentrations of Na^+ inside the cell is much lower than that outside the cell. Potassium (K^+) on the other hand is found in high concentrations inside the cell compared to the surroundings. Thus there is a tendency for Na^+ to move into the cell from outside and for K^+ to move out. Although membranes are fairly resistant to passive diffusion of ions some leakage would take place tending to equalize concentrations inside and out. The differences in ionic concentrations maintained however by a pumping system at the membrane which pumps Na^+ out and K into the cell. Energy is required for pumping of ions against the concentration gradients since low temperature or inhibitors or respiration allow equilibrium to be reached. The necessary energy comes from the standard energy yielding reactions of cells i.e., the hydrolysis of ATP and the enzyme ATPase is in the membrane itself.

The tendency for sodium to move into the cell that is in the energetically favourable direction has been utilized for the transport of various sugars and amino acids into cells. For example the transport of glucose is dependent on Na^+ and is coupled to the energetically

unfavourable movement of glucose. The active transport of Na^+ out of the cell by the energy dependant pump maintains the Na^+ concentration gradient necessary for the movement of both Na^+ and glucose into the cell (Fig. 2.14).

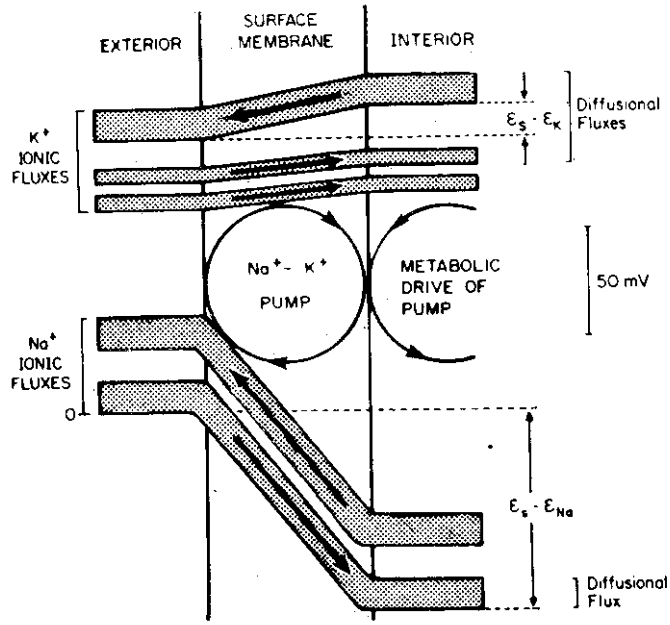

Fig 2.14 Active and passive Na^+ and K^+ fluxes through the membrane in the steady state. The ordinate is the electro-chemical potential of the ion ($\varepsilon_s - \varepsilon_k$ for K^+, $\varepsilon_s - \varepsilon_{Na}$ for Na^+). The abscissa is the distance in the vicinity of the membrane. The width of the band indicates the size of that particular one-way flux. Passive efflux of Na^+ is negligible and is not shown.

2.7 SUMMARY

In Part-II of Plasma membrane, artificial model systems, modifications, functions, membrane fluidity, membrane fusions and mechanism of transport are discussed. liposomes, discovered by Bangham is a special type of lamellar phase in which the water is self contained and the lipid molecules are arranged in bimolecular layers attached by their non-polar interfaces. They are excellent vehicles to carry foreign particles hence they are of immense value in medicine. Plasma membrane exhibits the phenomenon of phagocytosis and pinocytosis and is capable of undergoing several modifications like microvilli, desmosomes, myelin sheath etc. Besides selective permeability, it carries out several functions such as cell protection, formation of cytoplasmic organelles, maintaining balance of osmotic pressure and regulation of excretory material etc. By several experiments, it has been possible to prove membrane fluidity and membrane fusion. The mechanisms of transport may be passive or active. In passive transport, no energy is expended while in active transport energy is expended as the movement is against a concentration gradient.

2.8 MODEL QUESTIONS

1. Describe the structure and functions of the plasma membrane
2. Write about the artificial model systems and discuss the importance of liposomes.
3. Write an essay on modifications of the plasma membrane
4. Write short notes on:
   i) Liposomes
   ii) Pinocytosis and Phagocytosis
   iii) Capping Phenomenon
   iv) Cell fusion experiments
   v) Passive transport
   vi) Active transport

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Prof. N. Lakshmi

UNIT - I

Paper-VII : CELL BIOLOGY

Lesson 3

ENDOPLASMIC RETICULAM & GOLGI COMPLEX

3.1 ENDOPLASMIC RETICULAM

i) Introduction

In the cytoplasm of almost every kind of cells there exists an elaborate membrane system that form closed compartments in structural continuity. Porter was the first who called it endoplasmic reticulam since the membranes form a net work and endoplasmic in location. It forms a part of the endomembrane system which consists of the nuclear membrane, the rough and smooth endoplasmic reticulam and golgi complex.

ii) Electron micrographic structure

Electron micrographic studies revealed that endoplasmic reticulam or ergastoplasm is a membrane limited cisternal (sac like) system extending to varying degrees from the nuclear membrane on the inside of the plasma membrane. On the outside of the cell and forms an important part of the cell for the manufacture of cellular products. The membrane is trilaminar, the outer nuclear membrane is continuous with endoplasmic reticulam and when a cell divides the new

nuclear membrane is derived in part from the nearby remains of the endoplasmic reticulam. The thickness of the membrane is 50-60 μm. The endoplasmic reticulam is of variable morphology and can be present in varying amounts. The morphological forms of ER are cisternae (sac like) 40-50 mμ thick, tubules 50-100 mμ thick and vesicles 25-500 mμ (Figs. 3.1 and 3.2).

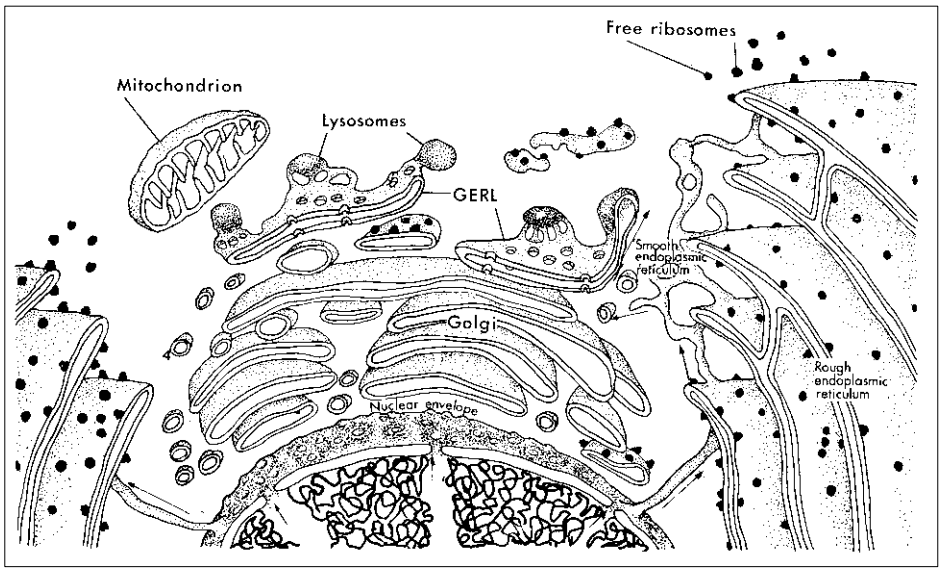

Fig 3.1 Three-dimensional diagram of the endomembrane system of the cell. The nucleus with its chromosomal fibrils shows interchromatin channels (arrows) leading to nuclear pores. Note the double-membrane organization of the nuclear envelope. Cisternae of rough endoplasmic reticulum are interconnected and have ribosomes attached to the outer surface. Some of these cisternae are extended by tubules of smooth endoplasmic reticulum. The Golgi apparatus shows the GERL region. The large arrows indicate the probable dynamic relationship of the portions of the endomembrane system.

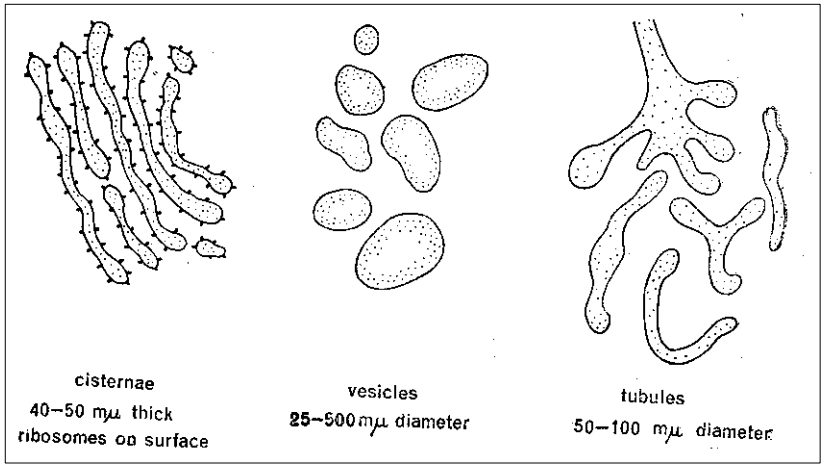

Fig 3.2 Cisternae, tubules & Vesicles of Endoplasmic reticulam.

In parotid cells from salivery gland of mouse, the sac like endoplasmic reticulam is much flattened like collapsed baloons with the individual cisternae arranged in parallel semicircular aggregates. In some the cisternae are more expanded and appear to form a continuously branching and interconnected system some cells may have substantial amounts of endoplasmic reticulam localized in given areas while being free of them in other areas.

The Smooth & Rough Endoplasmic reticulam

The membranes of the Endoplasmic reticulam (ER) are of two kinds, rough and smooth. Both may be present in the same cell e.g.:- Mammalian liver cell. The presence of either is an indication of the particular synthetic capacity of the cell, the rough endoplasmic reticulam with the attached ribosomes is well developed in the basophilic regions of the cytoplasm and found in great abundance in cells actively engaged in protein synthesis. But the synthesizing capacity is not solely a property of the membrane itself but rather is a complex process involving the co-operative interplay of nuclear membrane ER and ribosomes. The ribosomes are attached to the membrane by their 60s larger sub unit. In rough ER two proteins ribophorins-I & II are present. These are transmembrane glycoproteins that may correspond to the ribosomal binding sites. The smooth ER in liver is related to glycogen deposits and peroxisomes and is devoid of the two proteins ribophorins I & II.

iii) Functions of Endoplasmic reticulam

Numerous functions are attributed to endoplasmic reticulam. The functions of the endoplasmic reticulam are based on investigations on subcellular fractions isolated through centrifugation. During the process of isolation the endoplasmic reticulam is broken into smaller membrane fragments which sometimes reseal into vesicles called **microsomes**. Detailed biochemical studies on these membrane vesicles or microsomes showed the presence of various enzymatic activities. The functions of ER are summarized as below.

a) Intracellular circulation: Endoplasmic reticulam provides a system of communication in the cell which permits segregation of the products formed and channels for the transportation of the products either to the other part of the cell or to the outside.

b) It contributes to the mechanical support of the cytoplasm, has osmotic properties and is in involved in intracellular exchanges between the matrix and the internal cavity. There exchanges are brought about by diffusion and active transport in which carriers and permeases may be involved (Exchange of ions and any other fluid). In liver there are about 11 square meters of membrane per milliliter available for exchange.

c) Ionic gradients and electrical potential may be generated across the membranes and conduction of intracellular impulses have been postulated in the case of sarcoplasmic reticulam.

d) The smooth endoplasmic reticulam is involved with the synthesis of lipids, lipoproteins and glycogen. In liver, it is concerned with lipid and cholesterol metabolism and may be markedly increased in amount by the administration of lipid soluble drugs such as phenobarbital. It is involved in breaking down of glucose 6 phosphate into glucose and phosphate (glucogenolysis) by the enzyme present in SER.

e) The SER is concerned with the synthesis of steroid hormones in cells of testes and adrenal glands while it is concerned with the secretion of chloride ions in certain cells lining the mammalian stomach and gills of the fishes.

f) Another important function of SER is detoxification of many endogenous and exogenous compounds. The main principle of detoxification of chemicals is by converting hydrophobic substances into water soluble ones. Most of the drugs, pesticides, toxins and other chemical pollutants are generally hydrophobic compounds, so they try to accumulate in body fats. The reactions involved in detoxification include hydrolysis, reduction or conjugation. The oxidase enzyme systems for detoxification contain an important element cytochrome P 450. This is an iron containing compound having a special property of reducing its form, when it binds with the substrate. This reduced form can again absorb light at 450 nm. When any compound say drug is attached with the cytochrome P 450, the iron (Ferric Fe^{+++}) present is reduced to ferrous (Fe^{++}) with the help of NADPH dependent cytochrome P 450 reductase. Now the reduced cytochrome P 450 will bind oxygen. One atom of oxygen is used to oxidise the substrate from ferric to ferrous state and the other atom of oxygen is used to oxidise the drug with the liberation of the molecule of water.

This enzyme system is also employed in the oxidation of steroids and fatty acids. The constant habitual users of drugs for example use of sedative Phenobarbital increases the synthesis of enzyme oxidise present in liver and SER resulting into the decrease in effectiveness of other drugs like antibiotics, steroids, anticoagulants, narcotics etc. In addition to cytochrome P 450, cytochrome b_5 has been employed for the desaturation of fatty acids. Cytochrome P 448 tries to metabolize polycyclic hydrocarbons. All these elements are present in SER.

g) Endoplasmic reticulam plays an important role in cell division. In early part of cell division the nuclear membrane becomes fragmented into small vesicles and some large lamellar units. These move towards the poles and to the periphery where they are indistinguishable from the elements of ER. During telophase, the elements of ER migrate around into the regions around the chromosomes which are grouping at the poles and thus a part of the new nuclear membrane is developed by the elements of the endoplasmic reticulam.

h) The rough endoplasmic reticulam play a significant role in protein synthesis, protein transport, in functioning of the signal sequences and in protein processing.

3.2 GOLGI APPARATUS OR GOLGI COMPLEX

i) Introduction

In 1898, by means of a silver staining method Camillo Golgi discovered a reticular structure in the cytoplasm of nerve cells. In the next quarter of century, GA was described in different types of cells and in different animals. However, in 1923 a controversy about the existence of GA arose. No cell constituent has been the subject of so much research and discussion. However, the use of electron microscopy provided a distinct image of this membranous structure and could thus be studied in detail.

ii) Ultra Structure

Golgi complex is a differential portion of the endomembrane system which is morphologically very similar in animal and plant cells. However, they differ structurally in thickness of cisternae and in their protein and phospholipid composition. Further, plant dictyosomes have intercisternal elements which are absent in animal dictyosomes. Plant dictyosomes are usually more discrete while they were more compact and can be easily recognised as aggregate arrays in animal dictyosomes. It is spatially and temporally related to the ER on one side and to secretory vesicles leading to the cell membrane on the other.

There is variation in the shape of the Golgi apparatus from one cell to another and in the same cell, there is variation with the functional stage. It appears in two principal forms.

a) localized form
b) diffused form

When the dictyosome units are separated and scattered throughout the cell this form is known as diffused form. But these concentrate to form the localized form.

The GC is more often discontinuous, readily stained by osmium and silver containing dyes and more compact in morphology. It consists of parallel array of membrane enclosed flattered cisternae (ranging in number from 3.20 depending on the cell type) arranged in straight or curved bundles with numerous small vesicles 400-800 A^o in diameter clustered at the ends of cisternae. The cisternae are similar to the smooth endoplasmic reticulam but they differ in thickness 60-70 A^o (ER 40-50 mμ) and they are free from the attached ribosomes and are surrounded by zone in which the organelles are excluded. Each dictyosome is polarized with a proximal or forming face (convex or cis region and close to the nucleus) and a distal or maturing face (concave or trans). At the forming face are the vesicles and tubules that converge forming a fenestrated plate. The vesicles originate from the cisternae by pinching off process (Fig. 3.3).

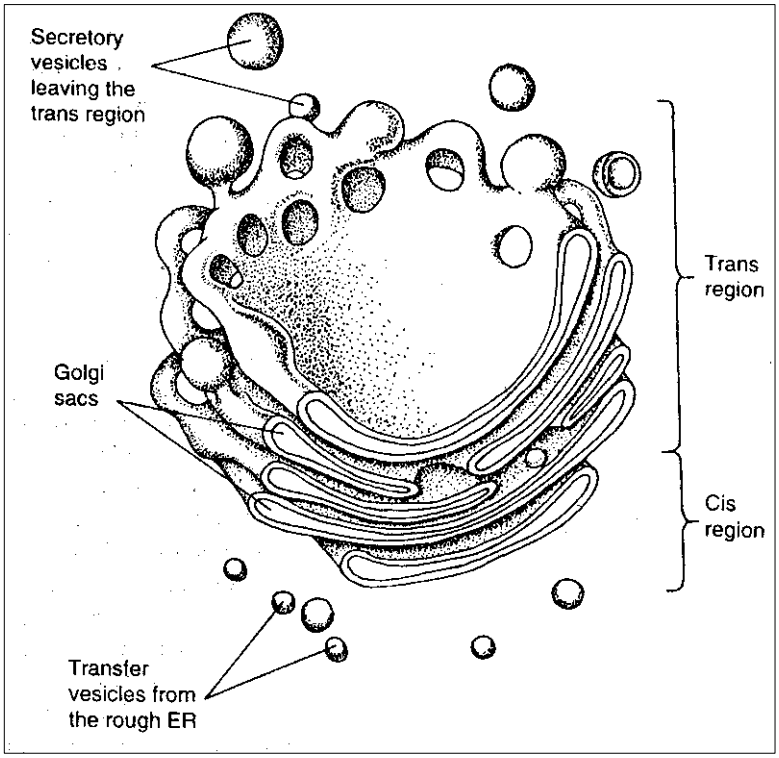

Fig. 3.3 A three-dimensional model of the complex of Golgi vesicles in a secretory cell. The transfer vesicles, which have budded off the rough ER, fuse with the cis membranes of the Golgi complex. The secretory vesicles that bud off the sacs on the trans membranes become, in acinar cells, the zymogen granules.

The Golgi complex or body is often called traffic police of the cell controlling the packing of proteins and vesicles to proper places.

iii) Chemical composition

Golgi Complex (GC) isolated from rat liver was made up of 60% protein & 40% lipid. The enzymes that appear most concentrated in Golgi fraction are thiamine pyrophosphatase and several glycosyl transferases that transfer oligosaccharides to the glycoproteins. Another enzyme unique for GC is Nucleosidediphosphatase. This enzyme may be present in some cases in ER some of the enzymes like 5 nucleotidase, Glucose 6 phosphatase, cytochrome oxidase and uricacid oxidase are common to GC, ER and plasma membrane. The enzyme glucansynthetase helps in carbohydrate metabolism.

iv) Functions

a) The Golgi Complex may be considered as an intermediary compartment interposed between the ER and the extracellular space through which there is continuous traffic of substances (i.e. fluids, macro molecules, cell wall units and other cell constituents). This traffic also involves the flow and differentiation of membranes and a rapid turnover. (Every 20-40 mi in dictyosomes of liver the cisternae are released at the rate of 1 for every few minutes).

b) One of the major functions of Golgi is the glycosidation of lipids and proteins to produce glycolipids and glyco proteins. Glycosyl transferases present in Golgi bodies carry out the glycosylation reactions. Sometimes the sugar residues are attached to protein chains after transporting it to Golgi complex. The proteins founded by membrane come out of Golgi complex are either stored as zymogen granules till a suitable signal helps to remove them from the cell. Sometimes, these vesicles are discharged immediately from the cell.

c) The Golgi complex is also involved in the secretion of proteins from the cell. A clear relationship between the Golgi system and membranes of the ER in secretion of proteins is evident. Some of the digestive enzymes to be secreted pass from their site of synthesis on the rough endoplasmic reticulam into the cavity of ER system, from there they are transported by the process thought to involve fusion of ER membranes with GC into the GC itself. The proteins are then concentrated into the vesicles which pinch off from the cisternae and ultimately fuse with the plasma membrane discharging their contents to the outside of the cell (Exocytosis). Glycoproteins can also be secreted in this way Insulin biosynthesis is a good example of molecular processing of secretion.

d) **Membrane recycling of secretory cells:** During secretion process, through exocytosis there will be considerable expansion of plasma membrane. So the cell must either degrade the extra membrane components or recycle them by endocytosis to maintain a constant membrane area. The bulk of these membrane components is recycled to the region of the GC where they fuse with the associated membrane. The recycling of the membrane is evidenced by the immuno fluorescent technique adopted by Willingham *et al*., (1984).

e) **Role of GC in cell wall formation:** The formation of cell wall in plant cells clearly depends on the activity of GC. Following mitosis vesicles from GC move to the region where the new walls will form and they fuse with each other. As a result of this fusion, two sheets of membrane form with the former contents of Golgi derived vesicles between them. The fused membrane then become the plasma membranes of the newly formed cell wall and the material between them form the matrix of the new cell walls and the middle lamella. Subsequent wall growth involves the fusion of vesicles containing polysaccharide material with the plasma membrane and the resulting deposition of material consisting of pectin and hemicellulose into the wall matrix. Some of the enzymes necessary for the synthesis of these cell wall constituents are believed to be constituents of GC. We can see therefore that GC is involved not only in synthesis and transport of wall material but also in the formation of plasma membrane itself. In animal cells also the GC appears to be contributing material to the expanding plasma membrane.

f) The Golgi complex also plays a role in the transformation of a spermatid into a mature spermatazoan.

g) In oogenesis GC is closely associated.

v) Origin: There are two views regarding the origin of GC. One view is that the endomembrane GC is derived from vesicles and tubules of endoplasmic reticulam. The second view is that GC is formed from pre existing dictyosomes by division.

3.3 SUMMARY

Endoplasmic reticulam and Golgi complex are the endomembrane systems of the cell. The membrane of endoplasmic reticulam are classified into rough and smooth type. Smooth endoplasmic reticulam are devoid of attached ribosomes. The structure is similar to that of cell membrane and the individual components are cisternae, tubules and vesicles. Several functions are attributed to ER such as intracellular circulation, mechanical support, synthesis of proteins, lipids, detoxification of many endogenous and exogenous compounds and glucogenolysis through the action of glucose-6-phosphate. ER has osmotic properties, ionic gradients and electrical potential may be generated across these membranes and conduction of intracellular impulses has been postulated in the case of SER.

The GC named after the discoverer is a discrete membranous structure consisting of parallel array of membrane enclosed flattered cisternae in straight or curved bundles with numerous small vesicles clustered at the ends of cisternae. Each dictyosome is polarised with cis and trans faces. At the forming face, the vesicles and tubules converge to form a ferestrated plate. The functions of the GC are determined by the enzymes associated with GC. GC can be compared to a traffic police regulating the traffic to proper places. The various functions of the GC include packaging and distribution of proteins and lipids, glycosidation of lipids and proteins, secretion of proteins in cell wall formation, role in spermatazoan formation and oogenesis.

3.4 QUESTIONS

1. Write about the structure and functions of endoplasmic reticulam
2. Give an account of structure and functions of Golgi Apparatus.
3. Write short notes on:
    a) Rough and Smooth endoplasmic reticulam
    b) Morphology of Golgi complex
    c) Functions of Endoplasmic reticulam
    d) Functions of Golgi complex
    e) Detoxification effects of ER

f) Cell secretion

3.5 REFERENCE BOOKS

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Prof. N. Lakshmi

UNIT - I

Paper-VII: CELL BIOLOGY

Lesson 4

MITOCHONDRIA & VACUOLES

OBJECTIVES

1. MITOCHONDRIA

i) Introduction

Mitochondria are present in the cytoplasm of all eukaryotic cells. They are absent in mature RBC, although they were present in immature erythrocyte. In some cells they can be seen as long slender rods but their shape and number are the characteristic of the cell in which they are present. In the living cells seen in tissue culture they appear to be in constant motion. Their visibility is increased by the vital stain janus green. They display active and passive motion and show changes in volume and shape that are related to their function. Swelling of mitochondria can be induced by Ca^{+}_{2}, various hormones and certain drugs. Mitochondria provide an energy transducing system by which the chemical energy contained in food stuffs is converted by oxydative phosphorylation into high energy phosphate bonds ATP.

ii) Morphology

The morphology of mitochondria can best be studied after fixation. They range in from spheres to rods to branching rods but in general they are rod shaped with a diameter of 0.5 μm and a variable length that may range upto 7 μm. The unicellular green alga *microsterias* has a single mitochondrion per cell where as the giant *Amoeba, Choas Choas* may have upto 500000. A mammalian liver cell 25 μm in diameter may possess as many as 1000, a kidney cell about 300 and sperm cell as few as 25. Green plants contain fewer mitochondria than animal cells. The distribution of mitochondria may be related to their function as suppliers of energy. Their orientation in the cell may be influenced by the organisation of the cytoplasmic matrix and vacuolar system.

iii) Mitochondrial Ultra structure

Altmann (1894) first described these granules by the name bioplasts. Later in 1897, Benda discovered these in the spermatogenous cells of animals and applied to them the term mitochondria. Most of the knowledge related to mitochondrial fine structure is due to the studies of Palade (1952) and Sjostrand (1955).

Each mitochondrion is bound on its outside by a smooth unit membrane about 6nm in thickness. Separated from it on the inside by a clear space of about 6-8nm is an inner membrane also of unit construction, which is variously folded into thin cristae extending into the cavity or matrix of the mitochondrion. The cristae may extend from wall to wall or only part of the wall way across the matrix of the mitochondrion (Fig. 4.1).

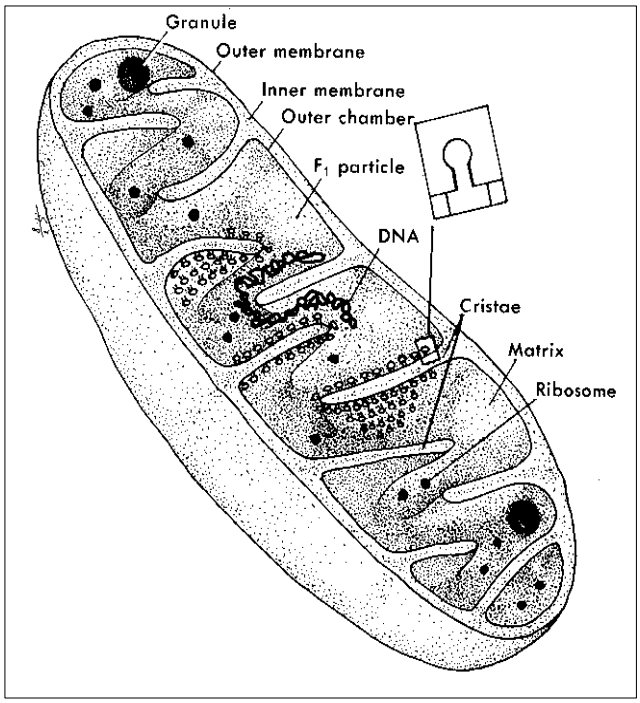

Fig 4.1 Three-dimensional diagram of a mitochondiron cut longitudinally. The main features are shown. Observe that the cristae are folds of the inner membrane and that on their matrix side they have the F_1 particles. The inset shows an F_1 partcile with the head piece and stalk.

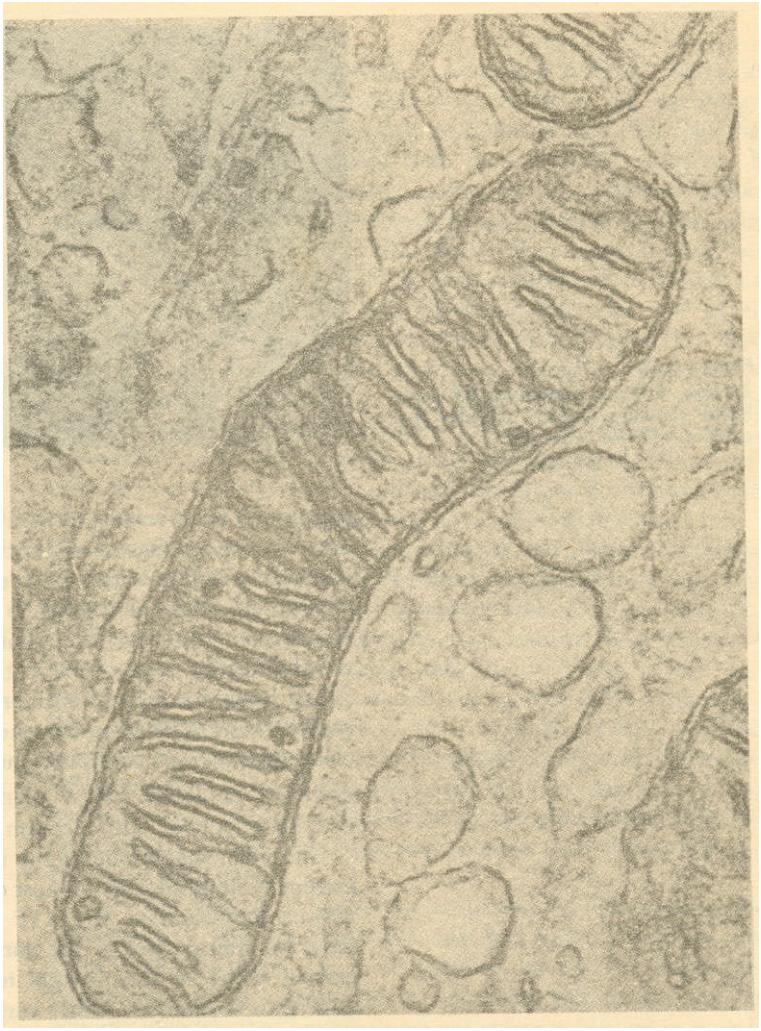

Fig. 4.1b. Highly magnified electron micrograph of a mitochondrion. the outer boundary of the mitochondrion is a double structure, with the inner layer being continuous with the inner cross membranes (cristae).

They may be relatively few in number leaving the majority of the mitochondrion occupied by the matrical substance or they may be so numerous as to nearly long the matrix with members (Fig. 4.1b).

In *Paramecium*, the cristae may be in the form of tubules rather than septa. These provide a greatly increased surface area on which sequential chemical reactions can take place. Enzyme complexes associated with cristae form an inseparable and integral part of the structure of the membranes. There is correlation between the number of cristae per mitochondrion and the chemical activity going on in the mitochondrion.

Staining with electron dense stains like osmium and potassium permanganate and then negative staining with phosphotungstic acid reveals added details of structure in the cristae. The inner surface of the cristae are studded with small particles of 0.5nm in diameter that are attached by short stalks. These so called elementary or F_1 particles are regularly spaced at intervals of 10nm

on the inner surface of these membranes. One square micrometer (1 μm^2) of membrane surface would contain approximately 4000 particles. These particles correspond to a special ATpase involved in coupling of oxidation and phosphorylation (Fig. 4.2).

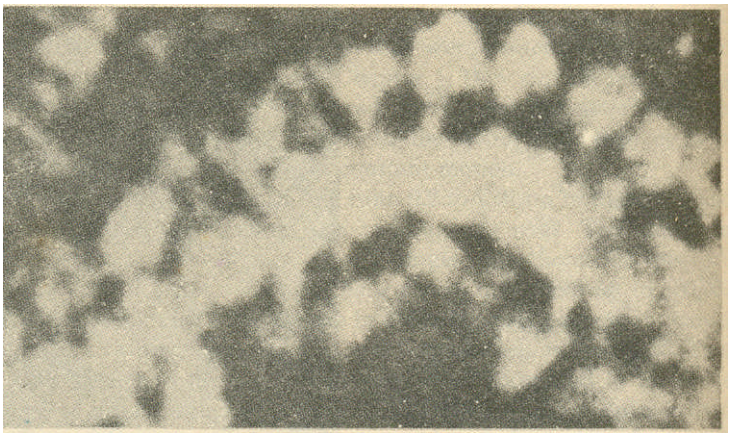

Fig 4.2 Portion of inner mitochondrial membrane showing stalked particles that contain ATP-ase activity.

The F_o F_1 synthase complex

A predominent protein in the mitochondrial membrane is the multi sub unit coupling factor - the enzyme that actually synthesizes ATP. An extremely similar enzyme complex located in the thylakoid membranes of chloroplasts and plasma membranes of bacterial cells. The coupling factor has two principle components, F_0 and F_1. In bacteria the integral membrane complex F_o is composed of 3 distinct polypeptides (a, b and c). Mitochondrial F_o is composed of similar a, b and c elements and depending on the species 2-5 additional peptides of unknown function. Attached to F_o is F_1, a complex of 5 distinct polypeptides (α, β, γ, δ & ε) with the composition α_3 β_3 γ δ ε. F_1 forms the knobs that protrude on the matrix side (Fig. 4.3).

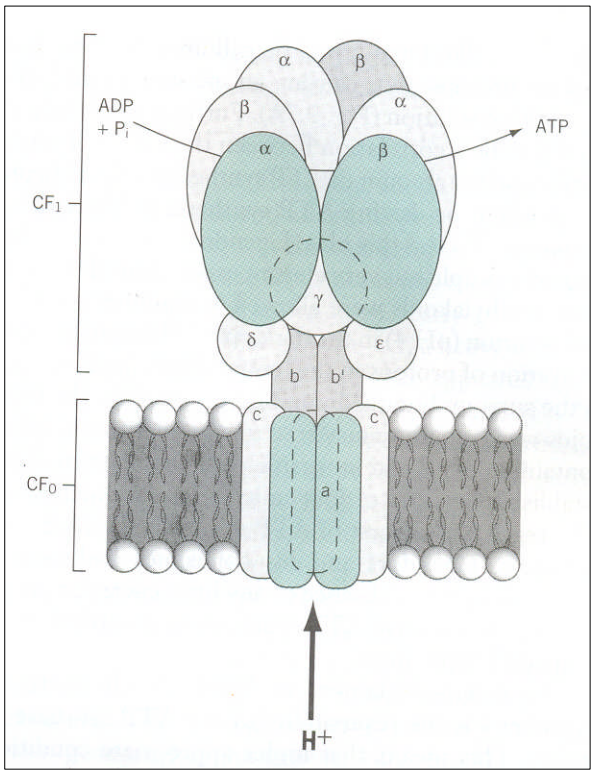

Fig. 4.3 A model of the chloroplast ATP synthase. GF_o is an integral membrane protein that forms a proton channel through the membrane. CF_1 is attached to the stromal side of CF_o and contains the active site for ATP synthesis, CF_1 consists of five different subunits with a stoichiometry of α_3, β_3, γ, δ, ε. CF_o consists of four different subunits with a probable stoichiometry of a, b, b', c_{10}.

of the inner membrane. F_1 can be detached from this membrane by mechanical agitation and water soluble. Within the mitochondrial matrix are ribosomes smaller in diameter than the cytoplasmic ribosomes (55S). They correspond to 70S ribosomes of bacteria rather than the 80S ribosomes of cytoplasm. It has been demonstrated that mitochondria also contain DNA and this indicates that atleast to some extent they can govern their own heritable qualities (semiautonomous).

iv) Mitochondrial genome

Mitochondiral DNA is circular and double stranded just like bacterial DNA. Guynene, cytosine content is higher in mt DNA, consequently the buoyant density is also higher. (1_1 706 g/m^1 in caesium chloride). It denatures readily and denaturation temperatures is higher. The amount of genetic information is not sufficient to provide specifications for all the proteins and enzymes present. So it codes for some structural proteins only. It does not have histones associated with it. In this respect it resemble the bacterial DNA. This DNA is able to code for ribosomal RNA, transfer RNA and a few proteins of the inner membrane. The differences between nuclear and mt DNA are given in table 4.1.

Table 4.1

Mitochondrial DNA	**Nuclear DNA**
1. It does not contain histones and has no nucleosomes	1. Itcontains histones and is packaged into nucleosomes
2. Here the reassocaition of mit-DNA is very rapid	2. Reassociation of nuclear DNA is not so rapid
3. It is generally circular except in *Paramoecium*, *Tetrahymena* and *Phaseolus*.	3. It is linear.
4. The genome size is small containing smaller number of genes	4. The genome size is large containing many genes.
5. The mitochondrial DNA consists of a mixture of opening circles and twisted or supercoiled circles	5. It does not contain any such structure.
6. This DNA preparations also contain some *Oligomeres* which appear as circular dimers.	6. No such circular dimers are found.
7. Repetitive sequence are rare in this type of DNA.	7. It is common in nuclear DNA.

The size and structure of mitochondrial DNA is variable. For example, the amount of mitochondrial DNA varies from 200-2400 kilobase pairs. Again, the recombination occurs within mitochondrial DNA causing genetic variation. Thus the mitochondrial DNA of higher plants is

more variable than that of other organisms. They have been studied in maize, Brassica, sorghum etc.

The main mitochondrial DNA of maize is circular with a size of 570 kilo base pairs having some other circular molecules of 67 kb, 250 kb, 253 kb and 503 kb. Of these 570 kb molecule is the master circle. This contains 6 repeated sequences of 1, 2, 3, 10, 12 and 14 kilo base. The reason for the origin of smaller circular molecules is due to the occurrence of recombination between these repeated sequences.

In the case of *Brassica campestris,* mitochondrial DNA is circular of 218 kb. It has two direct repeats of 135 kb and 83 kb. These two smaller circles are formed here due to recombination across the repetitive sequences and they have the capacity to recombine to form the master circle. Experimental data show that cucurbitaceae contains ten times mt DNA than that of *Brassica*.

Besides circular DNA, mitochondria of several plants like maize, sorghum, sugarbeet etc contain some small linear DNA molecules which have been detected through agarose gel electrophoresis. Of these two linear DNAs, S_1 (6.4 kb) and S_2 (5.4 kb) of maize have a great significance in regard to male sterility. Some times recombination takes place between S_1 DNAs and some of the circular mt DNAs resulting in linear chromosomes or DNA segments with S_1 or S_2 at the ends

Linear Chromosome

Due to this extensive recombination, a number of mitochondrial gene rearrangements have been found in maize. Some genes have been located on plant mitochondrial DNA. Some of the protein coding genes located on the mit-DNA are NADH dehydrogenase, ATP synthetase (3 sub units). Cytochrome-C-reductase, Cytochrome-C-Oxidase (3 sub units) apoprotein cytochrome b (COB), ribosomal proteins etc. There are genes for 26 S, 18 S and 5 S ribosomal RNA. The code CGG is generally used for arginine but in plant mitochondrial DNA, this code is used for trypotophan.

One interesting thing in plant mitochondrial DNA is that DNA can move between organelles. This DNA is known as promiscous DNA. For example, 12 kb region found in maize mitochondrial genome is homologous to chloroplast DNA. There is evidence that chloroplast gene sequences of 16S RNA, t-RNA and the large subunit of Rubisco are present in mitochondrial DNA. The method of transfer is not clearly known. Possibly this transfer may occur due to some transposable elements or due to some close association of cell organelles.

v. Mitochondrial membranes

The membranes and compartments of mitochondria can be separated by subcellular fractionation. The outer membrane contains NADH-Cytochrome-C-reductase which consists of a flavoprotein and cytochrome b_5. Monoamino-oxidase is the specific enzyme marker of this membrane. The outer compartment contains adenylate kinase and other soluble enzymes. The inner membrane carries all the components of the respiratory chain and of oxidative phosphorytation. The mitochondrial matrix contains soluble enzymes of the krebs cycle DNA, RNA and other components of the machinery for protein synthesis of the membrane.

vi) Functions

The mitochondria are the aerobic respiratory centres of the cell and are basically the energy converters. The carbohydrates and fats of the cell together with protein to a lesser extent are the sources of metabolic fuel used by the cell. Any molecule possesses stored energy in the bonds that link atoms together and when the bonds are broaken the energy is released. In the cells these bonds are successively broaken and energy is captured by suitable energy receptors rather than released as heat. The principal energy receptor is ATP and this molecule can give up energy to any part of the cell where work is being performed. ATP is infact cellular currency and the cell uses it to get things done in much the manner that we use currency for the purchase of things. The entire process of respiration requires the association of some 70 or more enzymes and the entry of O_2, ADP and phosphate and brings about the exit of ATP, H_2O and CO_2. The process is based on three co-ordinated steps: (a) The krebs cycle carried out by a series of soluble enzymes present in the mitochondrial matrix which produce CO_2 by decarboxylation and removes electrons from the metabolites (Fig. 4.4). (b) The respiratory chain or electron transport system which captures the pair of electrons and transfers them through a series of electron carriers, which finally leads by combination with activated oxygen to the formation of water. (c) A phosphorylating system tightly coupled to the respiratory chain which at three points give rise to ATP molecules.

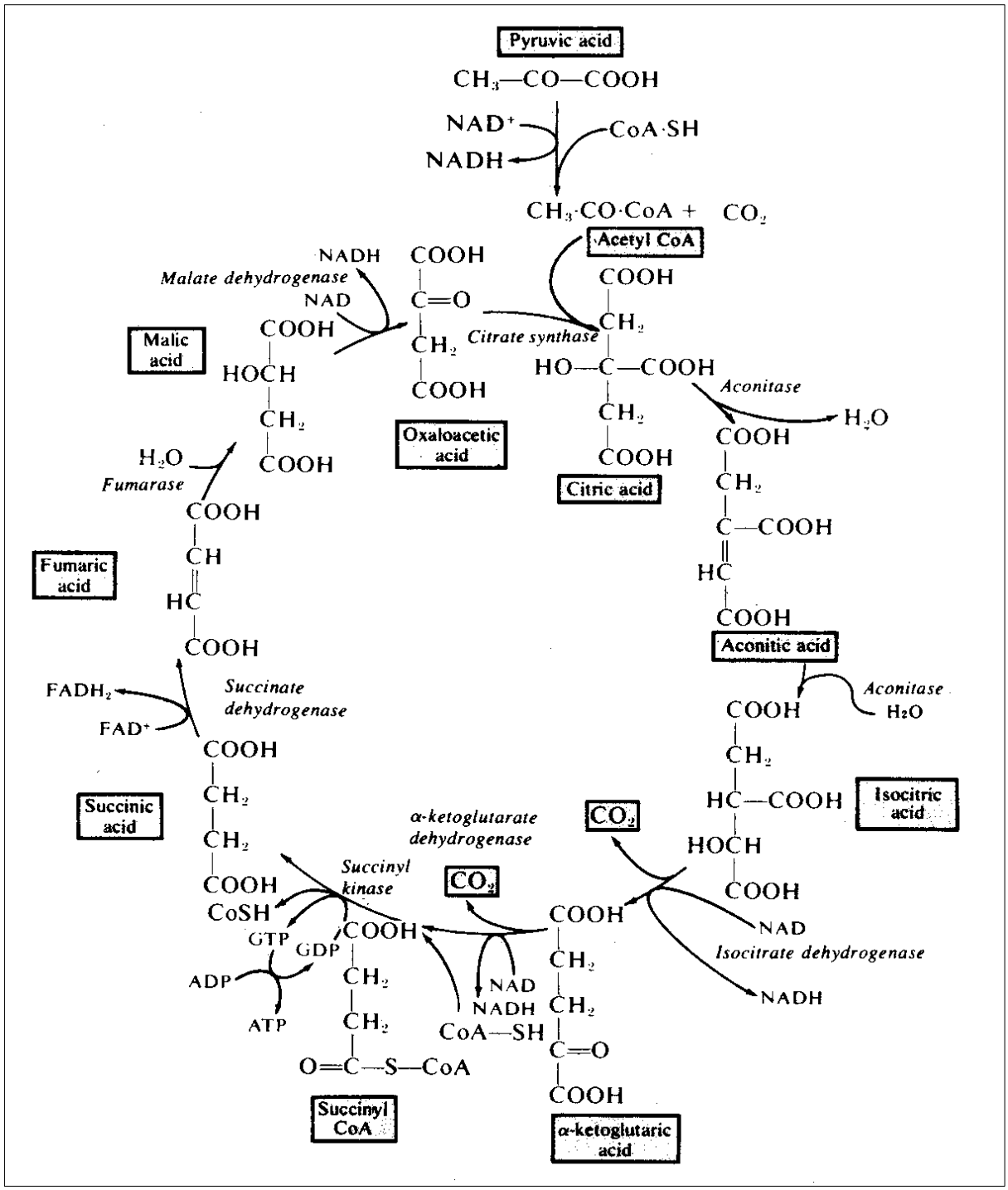
Pyruvic acid
CH3—CO—COOH
NAD+
NADH
CoA·SH
CH3·CO·CoA + CO2
Acetyl CoA
Malate dehydrogenase
NADH
NAD
Malic acid
Oxaloacetic acid
Citrate synthase
Citric acid
Aconitase
H2O
Fumarase
Fumaric acid
Aconitic acid
Succinate dehydrogenase
FADH2
FAD+
Succinic acid
Isocitric acid
α-ketoglutarate dehydrogenase
CO2
Succinyl kinase
GTP
GDP
ADP
ATP
CoSH
CoA—SH
Isocitrate dehydrogenase
Succinyl CoA
α-ketoglutaric acid

Fig. 4.4 Diagram of the Krebs or tricarboxylic acid cycle in mitochondria. The different enzymes and the steps of the reactions involved are represented.

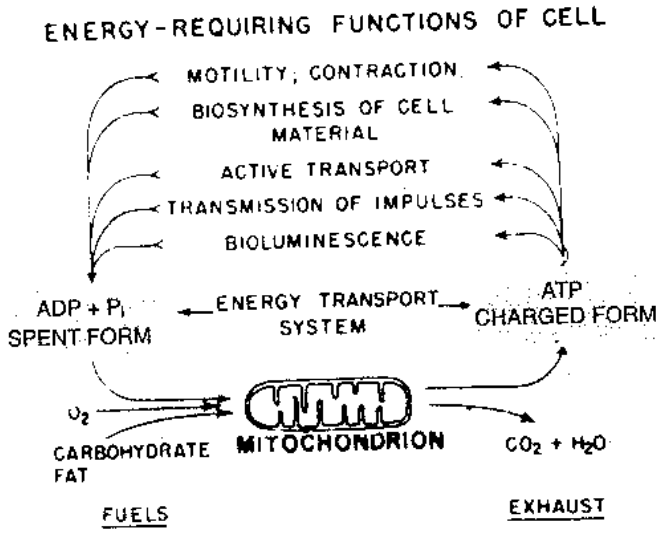

Fig. 4.5 Diagram showing that the mitochondrion constitutes the central "power plant" of the cell. The ATP produced is used in the various functions that are indicated.

Electron transport system

It starts with NAD^+ which is reduced to NADH. NAD^+/NADH has the most negative redox potential (Eo = 0.32 volts) and highest free energy (ΔG = 52.7 kcl/md). This is followed by a FMN^- (flavin mononucleotide) or a FAD (flavin adenine dinucleotide). Flavo protein coenzyme of 10 and a series of cytochromes ending with O_2 (Eo = 0.08 and ΔG=0). The respiratory chain also contains enzyme bound copper and iron sulphur proteins. The respiratory chain and the phosphorylating system are in the inner mitochondrial membrane. This is rich in proteins (60-70%) most of which are integral type. The components of the respiratory chain may be separated into multi molecular complexes of which lipids are essential for the activity. Four main components have been isolated.

Complex I or NADHQ reductase

It is the largest (mw > 500000) and has 15 subunits. It contains FMN and 6 iron sulphur centers. The NADH reaction site is at the M side (matrix).

Complex II or succinate Q reductase

It contains FAD and 3 iron sulphur centres. The complex transfers electrons from succinate COQ.

Complex III or Q H_2 - cytochrome C reductase

It contains cytochromes b, c and an iron sulphur protein. Cytochrome c is on the C side (Cytosol) and COQ is in the hydrophobic region of the membrane.

Complex IV or cytochrome-C-oxidase

It is a large complex with cytochromes a and a 3 and two copper atoms. As do other complexes it has a transmembranel orientation that is associated with the vectorial transfer of protons. In yeast mitochondria complex IV has 7 subunits of which the 3 larger ones are hydrophobic and synthesized on mitoribosomes.

The phosphorylating system is represented by the F_1 ATpase. This is a multipeptide complex with 3 main parts.

a) The F_1 particle or soluble ATpase.

b) A complex of very hydrophobic proteins (i.e. proteolipids) which represent the proton translocating proteins, and

c) A protein stalk that connects the two and connects the coupling factor.

By proper methods, it is possible to separate the F_1 particle and then to reconstitute the system (Fig 4.6).

The nature of the link between the respiratory chain and ATpase is unknown but there are two favoured hypotheses, the chemiosmotic and the chemical conformational.

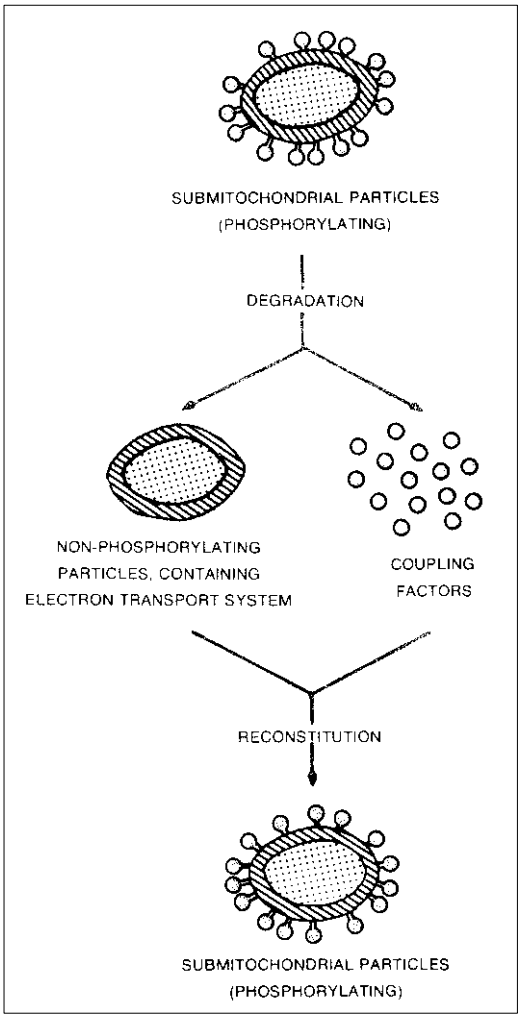

Fig. 4.6. Diagram showing the experiment by which the submitrochondrial particles, corresponding to the inner mitochondrial membranes, are submitted to urea to remove the coupling factor F_1, thus leaving non-phosphorylating particles. The lower part of the figure shows the subsequent reconstitution of a phosphorylating submitochondrial particle.

According to chemiosmotic, the ATpase synthesizes ATP under the influences of the vectorial field generated by the transfer of electrons through the respiratory chain. The effect operates at long range and direct molecular contact is not needed. The chemical conformational hypothesis postulates that the transfer of electrons originates i.e., conformational change in the proteins of the respiratory chain which by short range interaction transmits the signal to the ATpase that is needed for the synthesis of ATP. Mitochondria plays a significant role in the process of respiration. Other than this, they have role in yolk formation by accumulating iron containing pigments or protein molecules thus forming yolk bodies. They also play a role in sperm formation by forming a helix around the axial filament of the spermatid.

vii) Origin

There are various theories to explain the origin of mitochondria.

a) De no vo origin of mitochondria from a mixture of cell components like proteins, nucleic acid, lipids etc.

b) by growth and division of prexisting mitochondria

c) Origin of mitochondria through assembly of other membrane systems like plasma membrane, nuclear envelope, endoplasmic reticulam etc.

d) The symbiont hypothesis

The symbiont hypothesis postulates that mitochondria (and chloroplasts) originated from the symbiosis of a prokaryotic organism with a host cell which was anaerobic and derived its energy only through glycolysis. Mitochondria could be the result of a bacterium parasite residing in the cytoplasm of another cell. They are actually invaders which, in course of time not only established but also controlling the metabolism of the host cell.

Evidences in favour of symbiont hypothesis

(a) Circular double stranded nature of mt DNA just like bacteria.
(b) Mesosomes of bacteria are comparable to eristae of mitochondria.
(c) Ribosomes are small and similar to the bacterial ribosomes.
(d) In bacteria there is localization of respiratory chain in plasma membrane in mitochondria in inner membrane.
(e) Mitochondrial protein synthesis can be inhibited by chloramphinicol as in bacteria while cyclo-heximide inhibits that of cytosol.

So in evolutionary terms it is possible that a symbiontic relationship could have evolved into the present situation in which these organelles have only a certain degree of autonomy.

II. VACUOLES

Vacuoles are characteristic of plant cells. They are liquid filled areas of the cell delimited by a unit membrane called tonoplast. In young and actively growing cells small & several pro vacuoles) are present but as the cell matures, all these vacuoles coalesce together and form a single large vacuole which occupies nearly 90% the internal volume of the cell adpressing the nucleus to the plasma membrane and pushing the cytoplasm along with chloroplasts towards the exterior. This type of arrangement has two advantages. a) A ready exchange of gases can takes (2) Sunlight will reach the chloroplasts more readily as they are present at the periphery for the process of photosynthesis (Fig. 4.7).

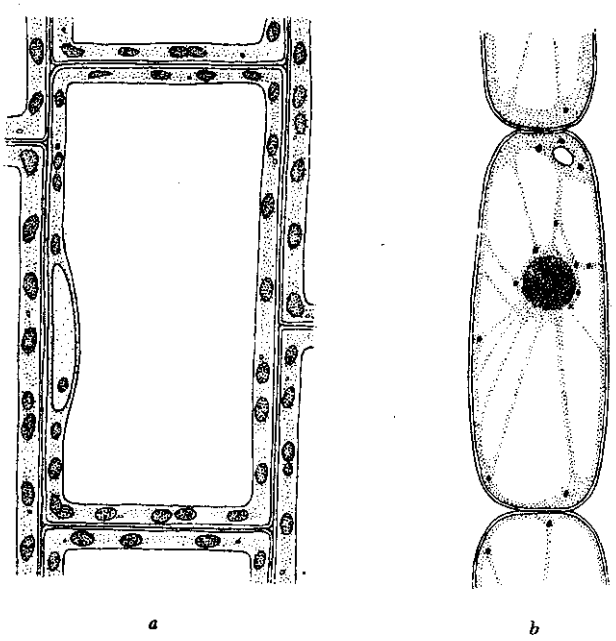

Fig. 4.7 Plant cells that reveal the existence of large sap vacuoles.
(a) onion skin cells; (b) stamen hair cells of the spiderwort, *Tradescantia virginiana*

The vacuole functions in the maintenance of turgor of plant cells and in providing an aqueous environment for the accumulation of water-soluble compounds. The tonoplast membrane like the plasma membrane is differentially permeable and hence can maintain concentration of materials very different from those found in the cytoplasm. The tendency of water to move into the hypertonic vacuole exerts pressure on the surrounding cytoplasm and hence on the cell wall. Thus vacuole helps in maintaining turger pressure of plant cells to remain relatively rigid but is also responsible for enlargement of the cell before the outer wall becomes too restricting. The vacuole also acts as a dumping place for waste and unwanted materials. High concentrations of salts, sugars and organic acids and water soluble pigments accumulate in vacuoles as large crystelline structures.

The red pigment of beet root and coloured pigments of many flowers are examples for the presence of pigments in vacuoles.

Lysosomal self-function of Vacuoles

The provacuoles are believed to be derived from the ER and possibly the Golgi, contain acid hydrolases similar to those of lybosomes. These are also associated with the tonoplast of the large vacuoles of differentiated cells. Occasionally, evidence of auto phagic activity can be seen inside the vacuole, various membrane components, including mitochondria and plastids can be detected in electron micrographs and these appear to be digested in the vacuole itself. In early stages of seed germination the vacuole splits to form membrane enclosed protein bodies which accumulate more of storage protein. The breakdown or digestion of food reserves to the developing embryo starts to take place in the vacuole and results from the hydralase activity.

4.3 SUMMARY

Mitochondria are the power houses of the cell. They are long slender rods of variable shape, size and number characterstic of the cell in which they are present. Each mitochondrion is a double membraned structure. The inner membrane is variously folded to form cristae on which the respiratory chain is located. They are aerobic respiratory centres of the cells and synthesize ATP molecules. ATpase present in F_1-F_o synthase complex multi subunit (coupling factor) is the enzyme that actually synthesizes ATP. Although there are several views regarding the origin of mitochondria the most evidenced view is the symbiotic origin of mitochondria. The genome of mitochondria which is a circular double stranded DNA with properties of prokaryotic DNA along with mitoribosomes similar to bacteria lends credance to this view.

Vacuoles are a characteristic feature of plant cell. Tonoplast is the unit membrane which surrounds the vacuole which maintains the turgor pressure of the cell. It is also a dumping place for waste and unwanted products. Large concentrations of salts, sugars, proteins and pigments accumulate in vacuoles. The red pigment of beet roots and coloured pigments of flowers are examples vacuoles also exhibit lysosomal activity by containing acid hydrolases inside.

In young and immature cells several provacuoles are present and the nucleus occupies a geometric centre. But in old and mature cells the nucleus is adpressed to the cell membrane and a single large vacuole is present. This arrangement is of value to the cell as there will be ready exchange of gases and facilitates easy photosynthesis as the chloroplasts are present at the periphery.

4.4 MODEL QUESTIONS

1) Describe the structure and functions of mitochondria.

2) Write short notes on:

   a) Plant vacuoles

   b) Electron transport system of mitochondria

   c) F_1 particles or F_1 - F_o synthase complex

   d) Chemiosmotic & Chemical conformational theories.

   e) Genome of mitochondria.

   f) Symbiont hypothesis

3) Mitochondria are the aerobic respiratory centres of the cell. Substantiate the statement.

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Prof. N. Lakshmi

UNIT - 1

Paper-VII : CELL BIOLOGY

Lesson 5

STRUCTURE AND FUNCTIONS OF CHLOROPLAST AND LYSOSOMES

OBJECTIVES

5.1 CHLOROPLAST

i) Introduction

Chloroplasts are the structures evolved by the cell through long ages of change for the trapping of light energy, its conversion into chemical energy and its storage in molecules derived from CO_2 and water. This process is known as photosynthesis and is initiated by the capture of light energy through absorption in the green pigment chlorophyll. The chloroplast is the cytoplasmic particle in which this process takes place. Their shape, size, distribution and number vary in different cells but are fairly constant for a given tissue. In higher plants they are discoid, 4-6 μm in diameter and number about 20-40 per cell. The size and number are genetically controlled. In polyploids they are larger in size and fewer in number. Chloroplasts multiply by division. The

quantity of light available causes chloroplasts to undergo changes in shape and volume by contraction or swelling.

ii) Ultra Structure

Electron microscope studies reveal that chloroplast is a structure of considerable complexity. It is a double membraned envelope inside of which is a complex internal membrane system which organise into a series of lamellar areas (grana or discs or thylakoids) which are connected to each other by extensions of the lamellar structures called thylakoid membranes (Figs. 5.1 & 5.2).

The grana granules are 0.3 - 1.7 μm and are embedded in the matrix. The intergrana areas are called the stroma. The grana can be visualized as many layered pieces of plywood or as stocks of coins lying in the less well organised stroma. Within the grana, the chlorophyll molecules are oriented in a monolayer sandwitched in between the layers of enzymatic proteins and intimately associated with phospholipids and carotenoids an arrangement that makes for efficiency not only for trapping of light energy but also for its conversion, conduction and utilization in photosynthesis.

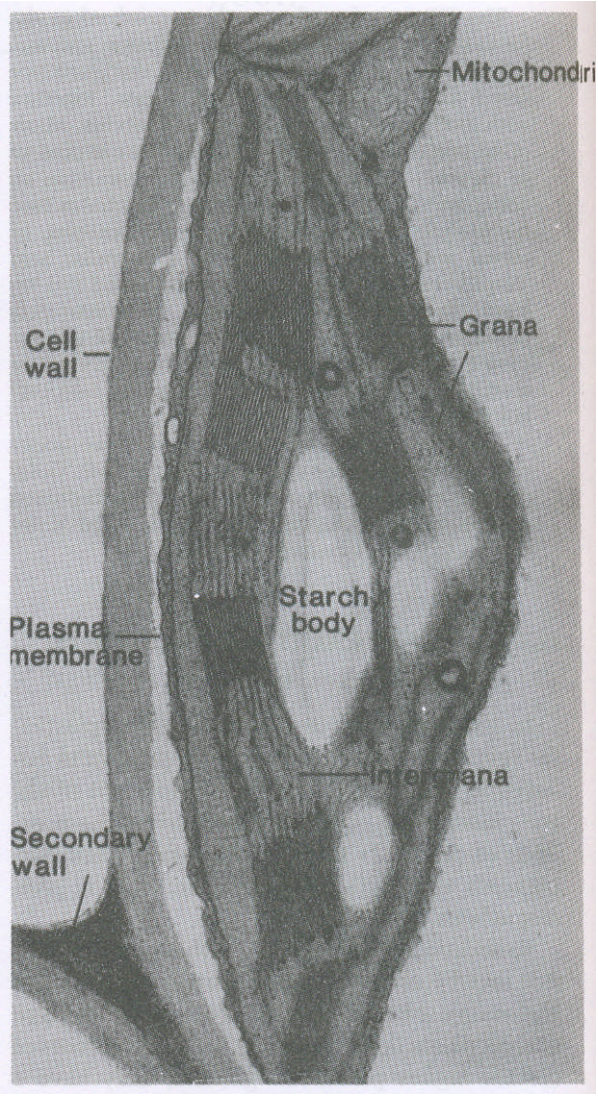

Fig 5.1 Longitudinal section of a chloroplast of *Linaria vulgaris* showing the grana and the intergranal thylakoids. (Courtesy of M. Cresti and M. Wurtz).

Electron microscopic examination of freeze fractured and freeze etched thylakoid membranes clearly demonstrates the presence of two major size classes of particles or sub units within the grana membranes. The larger of these approximately 18 nm in diameter and oriented toward that surface of the membrane facing the interior of the thylakoid, while the smaller subunit approximately 115A^{o} (15 nm) in diameter is a component of that half of the membrane whose surface faces the stroma.

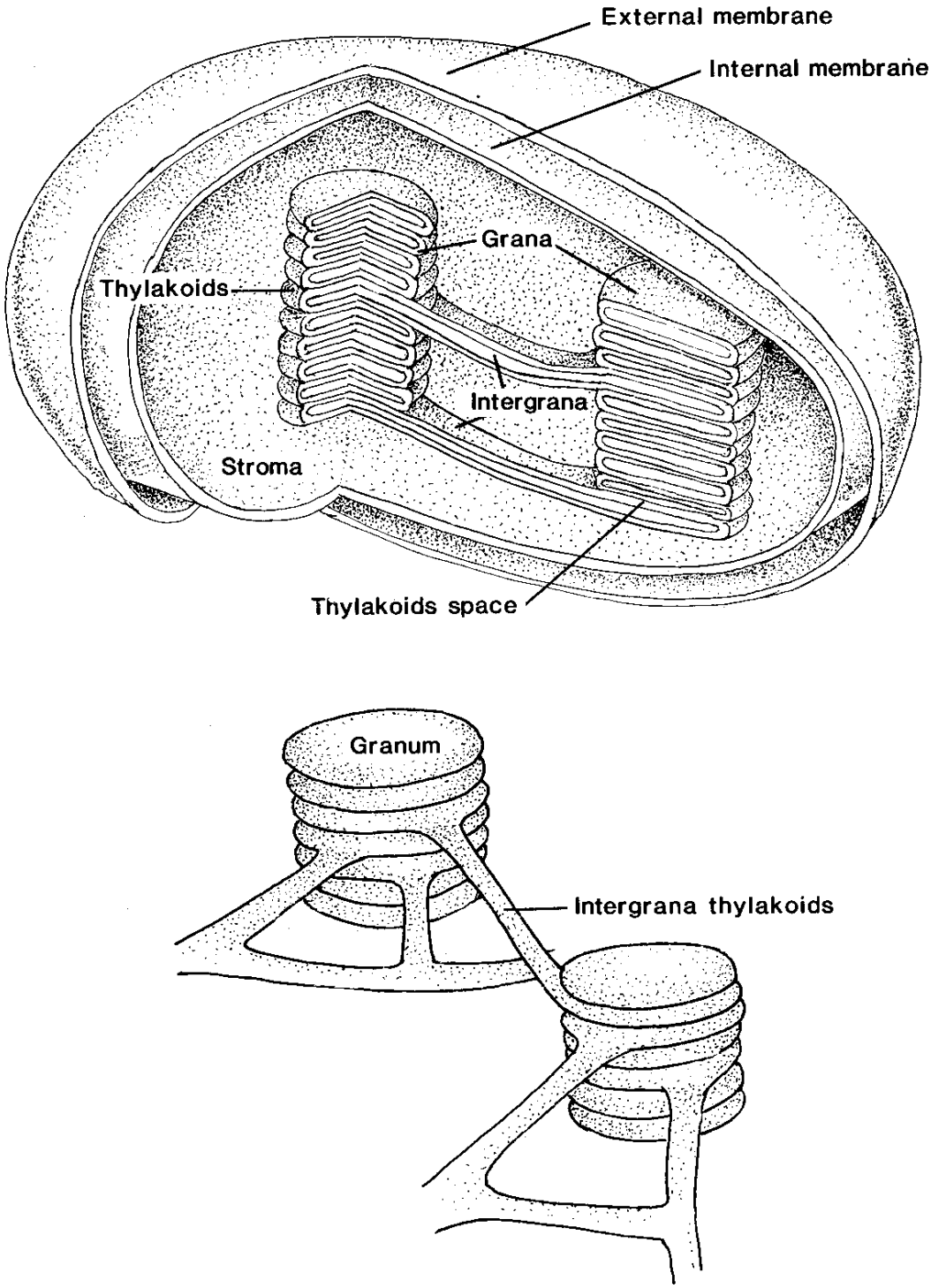

Fig. 5.2 Top, diagram of a chloroplast showing the mains structural components, Bottom, three-dimensional diagram of two grana with the stacked thylakoids and the unstacked ones that cross through the stoma of the chloroplast.

This structural asymmetry of the internal chloroplast membrane is related to the corresponding functional asymmetry. There is evidence that at least part of photosystem I occurs at the outer or stroma surface of the membrane and that photosystem. I activity is associated with the small particles while the larger particles are involved in photosystem II toward the inner facing surface (Fig. 5.3).

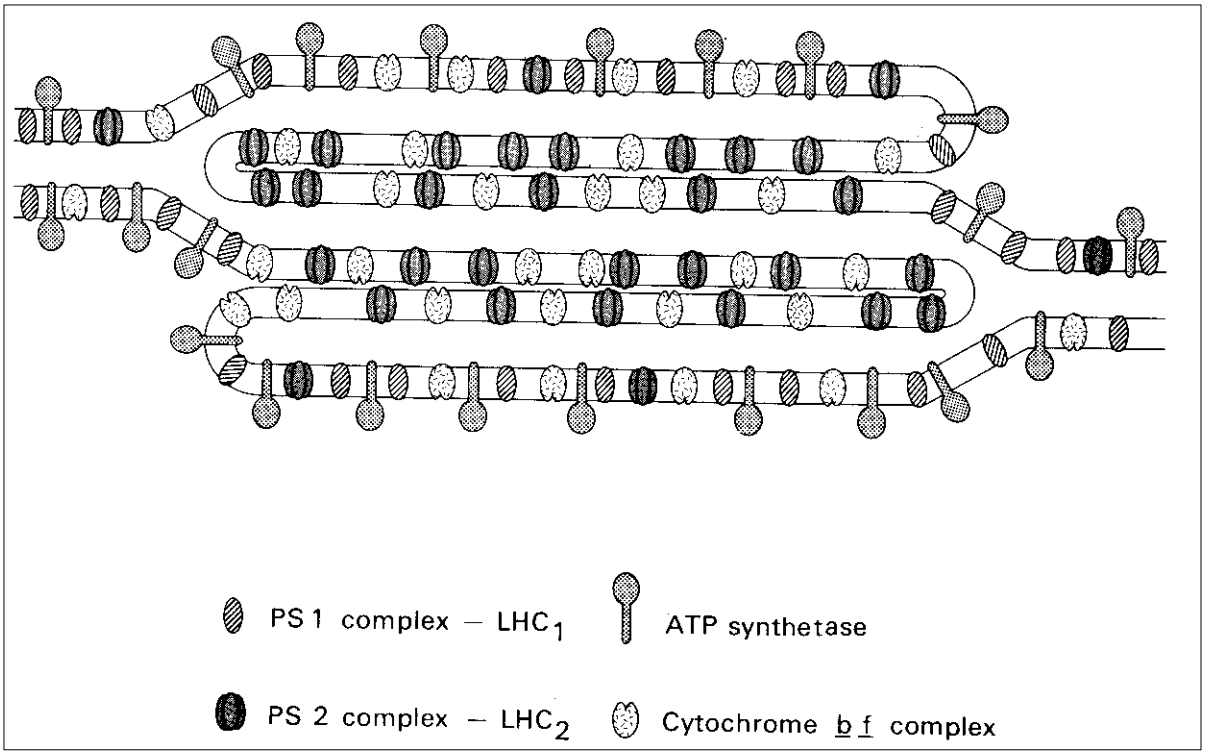

Fig. 5.3 Diagram showing the distribution f the main complexes within the thylakoid membrane both in the granal (stacked) and stromal (unstacked) regions. The different complexes are indicated at the bottom of the figure. Observe that the ATP synthetase is only present in the stromal surface of the grana and the stromal thylakoids. The stacked regions contain the other three complexes (Courtesy of J.M. Anderson and B. Anderson).

The stroma is a gel fluid phase (aqueous part of the chloroplast) containing dissolved salts and enzymes which catalyse the dark reaction of photosynthesis. It has DNA and ribosomes hence semi autonomous.

Chloroplasts may assume many forms and vary widely in number per cell in different plants. In algae such as **spirogyra** only a single spiral chloroplast is present in each cell. When the cell divides, it divides at the same time. In contrast a cell in the spongy part of a grass leaf may have 30-50 chloroplasts, their division is not correlated with cell division. In some brown algae the

stacked grana are missing in the chloroplast and instead long membranes running with in the chloroplast function as grana. In blue green algae definite chloroplasts are absent but they have loosely arranged membranes in the cytoplasme on which the photosynthetic pigments are layered only in bacterial cells photosynthetic capacity is unassociated with the membrane structure (Fig. 5.4). However, the vacuole like chromotophores of bacteria which are the photosynthetic units are bounded by membranes but little is known about the molecular disposition pigments. However, bacteria kept in dark loose their chromatophores and are no longer photosynthetic and this chromatophore is equivalent to chloroplast functionally but not structurally. Other bacteria will have beautiful membrane systems equivalent to grana but the membranes are chemosynthetic rather than photosynthetic (Fig. 5.5).

Fig. 5.4 Electron micrograph of a blue-green algal cell showing photosynthetic membranes. Particles in the membranes can be seen (Courtesy of Dr. S.C. Holt).

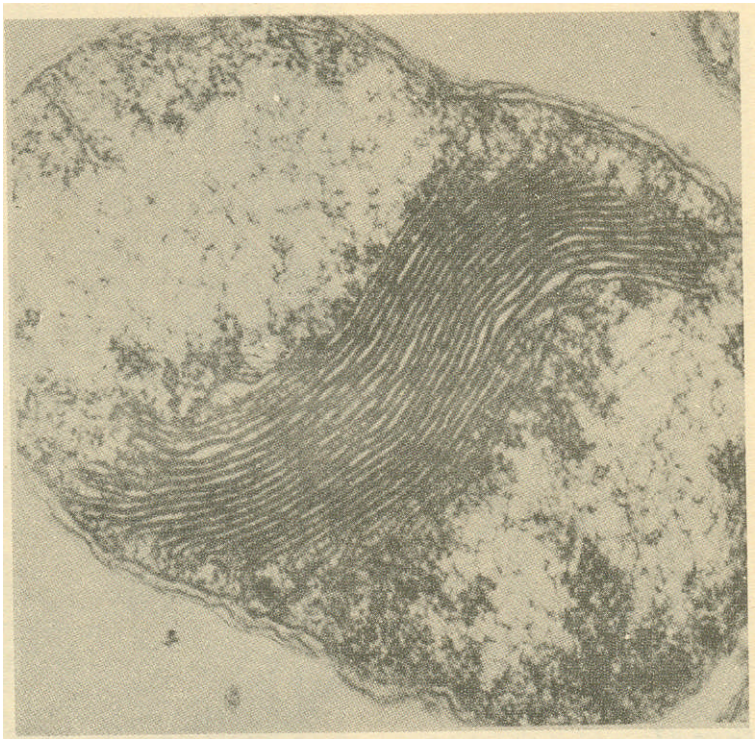

Fig. 5.5 Electron micrograph of the marine bacterium, *Nitrosocystis oceanus*. The elaborate membrane system is comparable to the photosynthetic membrane of plastids, but the former, however, is engaged in chemosynthesis, a process in which energy is obtained from the alteration of chemical compounds rather than from light sources. (Courtesy of Dr. S.Watson).

iii) Genome of Chloroplast

Ris & Plant first demonstrated DNA in the chloroplast in 1962. DNA of chloroplast is circular double stranded with an average length of 45 μm with 1,20,000 to 1,56,000 base pairs with molecular weight between 80-100 millions CP DNA (Chloroplast DNA) is without any association with histones 5 methyl cytosine is absent in CP DNA. The percentage of GC in the chloroplast DNA varies from 36-40% in different species. The reassociation rate of CP DNA with nuclear DNA varies from 10-20%. There are many copies of chloroplast DNA which is 200 copies or more. The replication of CP DNA takes place in a semiconservative way. In case of maize and pea, the replication starts at two loci having distance between them as 7,000 base pairs. The autonomous replication sequences, like ars sequences of yeast has been reported in the chloroplast DNA of tobacco.

The complete DNA sequence of chloroplast DNA has been determined incase of *Marchantia polymorpha* and tobacco. With the help of hybridization experiments of DNA-RNA, many protein coding genes, positions of many t-RNAs and r-RNAs of the chloroplast ribosomes have been identified. The tobacco chloroplast DNA has 155, 844 base pairs and code for about 146 genes. CP DNA of **Marchantia** has 128 genes with 121024 base pairs. CP DNA contains a pair of inverted repeats which encodes generally the r-RNA genes. There are some ORFs (open reading frames) with coding sequences beginning with a met codon and a stop codon at the end in the

chloroplast DNA. In tobacco genes for 23 S, 16 S, 4.5 S and 5 S ribosomal RNA and about 30 t-RNAs have been detected. Codes for some ribosomal proteins (rpl and rps) some subunits of RNA polymerase (rpo ABC) and some large submit of RUBISCO (rbc L) have been identified in chloroplast DNA of tobacco. Two genes for the polypeptides of PS I and eight for the polypeptide of PS II have been identified. Some herbicide resistant genes have also been located in the CP DNA. It has also been noted that some CP DNA contains genes which show homology with NADH dehydrogenase from human mitochondria.

iv) Ribosomes of Chloroplast

The ribosomes of chloroplasts are 70s and show more similarity with bacteria and blue green algae ribosome, subunits. Ribosomal proteins of the chloroplast are encoded both by the nucleus and the chloroplast DNA showing their interdependance. The structure of the chloroplast t-RNA shows more similarity with that of prokaryotic cells. With the help of two protein inhibitors cyclohexamide and chloramphinicol it has been noted that the synthesis of small subunit is inhibited by cyclohexamide and larger subunit by chloramphiniced. This shows that the small subunit is controlled by the nuclear gene and the large subunit by the chloroplast gene.

The large subunit RUBISCO was the first polypeptide synthesised among the chloroplast proteins. Besides this about 60 polypeptides have been synthesised by chloroplast.

v) Origin

Plastids like mitochondria do not arise de-no-vo but from other plastids by division and most likely at the proplastid or the under developed stage. In spirogyra plastid division is correlated with cell division but in higher plants the correlation is not evident. Just like mitochondria, chloroplasts also have their own DNA and ribosomes and the same suggestion has been made that they are symbiotic invaders of cyanophycean origin and during the course of evolution have taken over an essential part in cellular metabolism. They thus form somewhat independent units of inheritance within the cell eventhough their function and morphology is determined in part by nucleus. (the same details given in symbiont hypothesis of mitochondria are to be given here also except for the difference that mitochondria are of bacterial origin while chloroplasts originated from symbiosis of cyanophycean menebers).

vi) Function

Chloroplasts are the structures associated with the function of photosynthesis. In thylakoids, chlorophyll carotenoid molecules and a reaction centre are assembled forming two photosystems I & II. Each photosystem is associated with an electron transport system and with structural proteins. Lipids represent 50% of the thylakoid membrane. These include those involved in photosynthesis (i.e. chlorophyll, carotenoids and plastoquinone) and structural lipids.

Chlorophyll the main pigment is an asymmetrical molecule with porphyrin head composed of 4 pyrrole rings and forming a complex with mg atom. The molecule also has a long hydrophilic phytol chain. There are several types of chlorophylls *a, b, c, d* and *e* types, of these *a* & *b* are common in higher plants (Fig. 5.6).

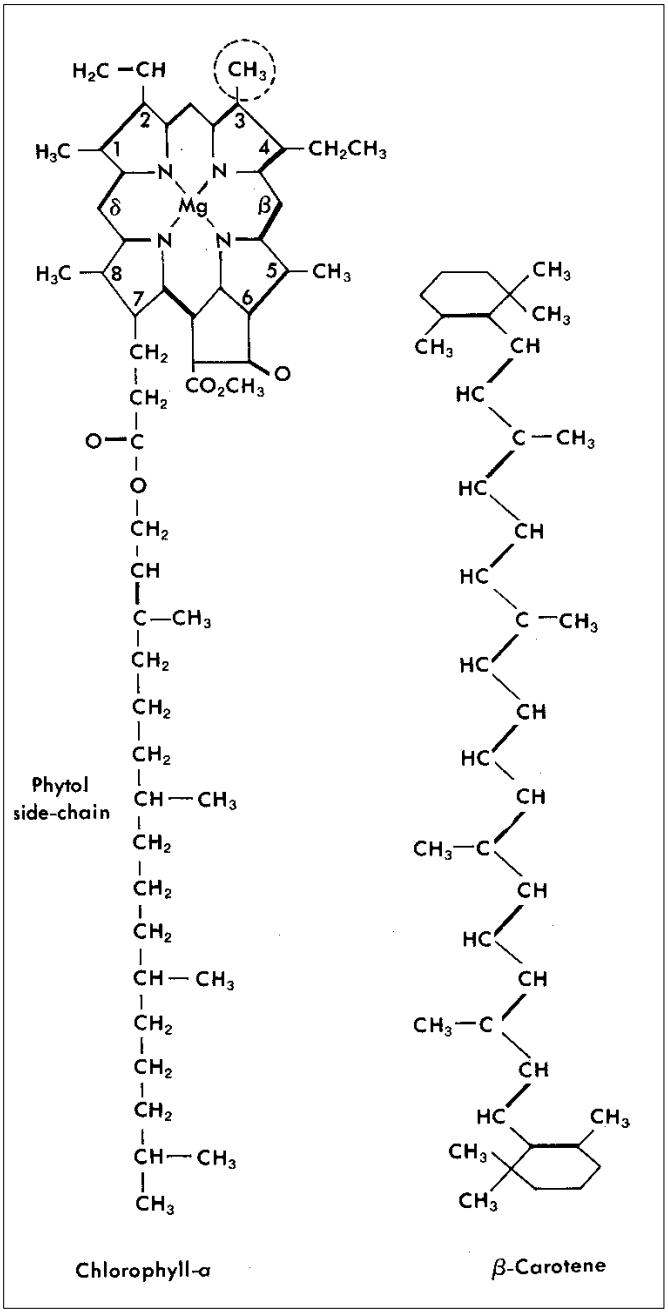

Fig 5.6. Structural formula of chlorophyll-a and β-carotene

This green pigment absorbs light at 663nm in the isolated condition. Another pigment called P 700 is bleached at 700 nm PS I & PS II complexes are present mainly in grana thylakoids. Chloroplasts have a phosphorylating coupling factor (CF_1) similar to that of mitochondria. The CF_1 particles are localized mainly in the outer surface of the stromal thylakoids.

Photosynthesis is a process through which the chloroplasts trap the light quanta (Photons and excitons) and transform them into chemical energy. Photosynthesis is in some ways the reverse of oxidative phosphorylation in mitochondria. The overall reaction of photosynthesis is

$$2n\ H_2O + nco_2 \rightarrow nH_2O + no_2 + (CH_2O)_n$$

Thus using H_2O as a hydrogen donar and CO_2 from atmosphere, carbohydrates are synthesized and O_2 released.

Photosynthesis consists of a photochemical reaction (Hill reaction) which occurs in the presence of light and a dark or thermochemical reaction. In the first reaction O_2 is released when the chloroplasts are exposed to light. In the second CO_2 is fixed and carbohydrates are formed. In the photochemical reaction, electrons flow from H_2O to $NADPH_2$ because electrons are boosted to high energy levels by absorbed light. In photosynthesis there are the photosystems, I & II that are excited at different wave lengths.

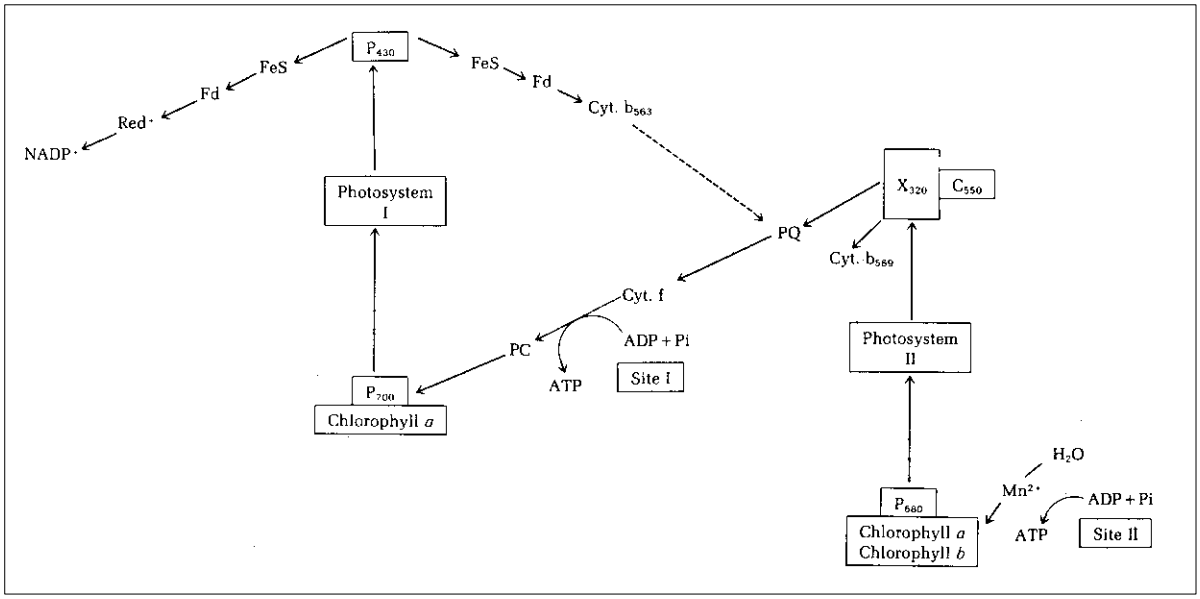

Fig. 5.7 Diagram showing the possible electron transport in chloroplasts. P 700 and P 680 are the photoexcited pigments of photosystems I and II. P_{430} and X_{320} are the primary acceptors of both photosystems; C550 is β carotene; PQ, plastoquinone; Cyt., cytochromes; PC, plastocyanine; FeS, sulfurerroprotein; Fd, Red^+- reductase. Sites I and II indicate the places where ATP is synthesized.

Photosystem I excited at 680-700 nm comprises 200 molecules of chlorophyll *a*, 50 of carotenoids and one of P 700. Photosystem II excited at 650nm contains chlorophylls *a* and *b*, only this second system is associated with the release of O_2 from H_2O. Both photosystems operate in a sequential and inter related fashion. In photosystem I two quanta of light trapped by P 700 boost electrons that reduce ferredoxin, a protein containing iron and sulphur. In turn ferridoxin transfers the electrons to $NADP^+$ reducing it to $NADPH_2$. The electrons in photosystem I are restored by the second system. Here the light quanta remove the electrons from H_2 of H_2O releasing O_2. The electrons are transferred to P 700 by a transport system which comprises several cytochromes and plasto cyanin (a copper containing protein) - two quinones - Vitamin K and plastoquinone are also included in the electron transport system. As in mitochondria the transfer of electrons is coupled with phosphorylation of ATP at the level of photosystem II (Fig. 5.7).

The dark or thermochemical reaction involves many steps which starts with the uptake of CO_2 and its reduction by $NADPH_2$ to form the various carbohydrates. In several steps of this complex cycle of reactions ATP is also used as an energy source of dominant compound is 3 phosphoglyceric acid which gives rise to glucose to phosphate from which come the various disaccharides and polysaccharides. The formation of phosphoglyceric acid depend on the initial enzyme carboxydismutase (Fig. 5.8).

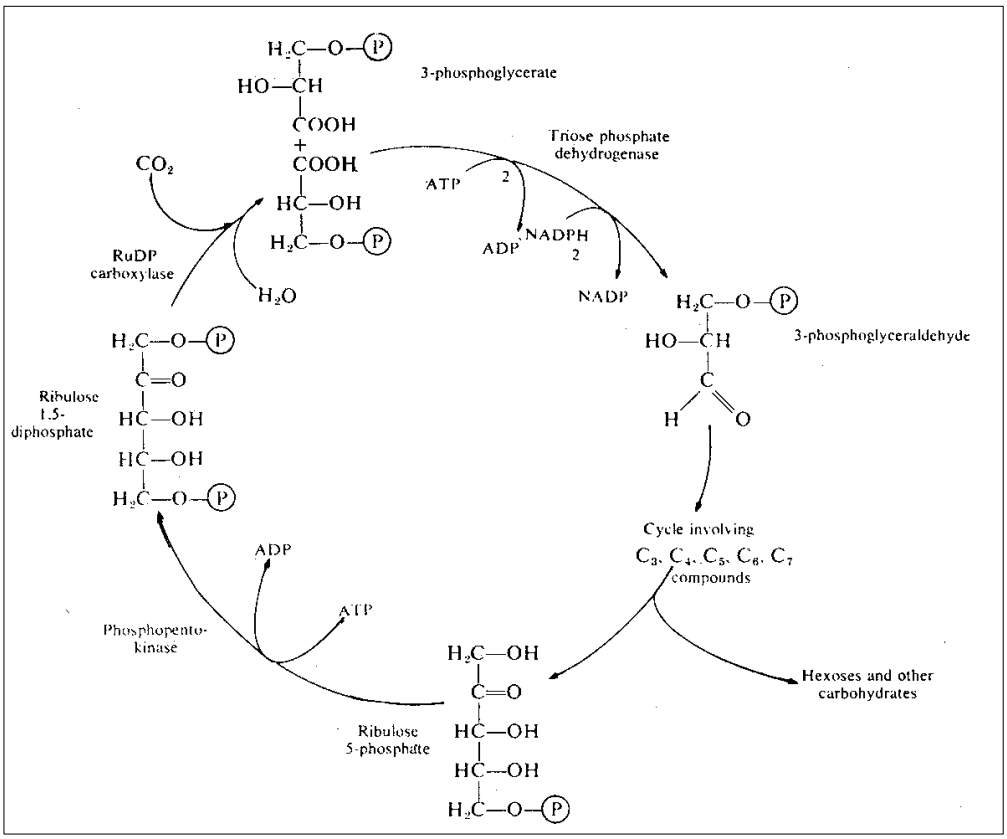

Fig. 5.8 Diagram of the Calvin or C_3 cycle of photosynthesis in which CO_2 is reduced and carbohydrates are synthesized.

All the enzymes of the dark reaction are soluble and are contained in the stroma of the chloroplast.

The chloroplast is a dual energy converter since the energy of sugars and ATP can be used in the cell in various ways.

5.2 LYSOSOMES

i) Introduction

Lysosomes are cytoplasmic organelles of all Eukaryotes that contain numerous (about 50) hydrolytic enzymes such as lysin, ribonuclease and phosphatase etc and in which the main functions are intracellular and extracellular digestion. The term 'Lysosome' was derived from two 'Greek words', the lyso (digestive) and 'soma' body. In 1949, these particles were isolated by De Duve and they have centrifugal properties between those of mitochondria and microsomes. The lysosomal enzymes are enclosed by a membrane homologous with unit membrane (accounting for their latency) and generally act at acid pH.

ii) Morphology

In electron microscope they can be recognised by using different cytochemical reactions. They vary in size 0.4 to 0.8 μm. Internal structure is variable, some have dense content, some others have a very dense outer zone with a less dense core and still others have cavities or vacuoles.

The most remarkable morphological characteristic of lysosomes is its polymorphism, particularly regarding the size of the particle and the irregularities in its internal structure. At present four types of lysosomes are recognised of which only the first is the primary lysosome, the other three may be grouped together as secondary lysosomes.

a) Primary lysosomes

These are storage granules and dense particles of about 0.4 μm surrounded by a single membrane. Their enzymatic content is synthesized by ribosomes in the endoplasmic reticulam and appear in the Golgi region. The formation of primary lysosomes can be blocked by puromycin.

b) The heterophagosome of digestive vacuole

The result from phagocytosis or pinocytosis of foreign material by the cell. This body which contains the engulfed material within a membrane shows a positive phosphatage reaction which may be due to the association with a primary lysosome. The engulfed material is progressively digested by the hydrolytic enzymes which have been incorporated into the lysosome. The extent of this digestion depends on the amount and chemical nature of the material and the

activity and specificity of the lysosomal enzymes. Digestion leads to products of low molecular weight which pass through the lysosomal membrane and are incorporated into the cell and to be used again in many metabolic path ways (Fig. 5.9).

c) Residual bodies

These are formed if the digestion is incomplete. In some cells such as **Amoeba** and other protozoa, these residual bodies are eliminated by defecation. In other cells they may remain for a long time and may be important in the aging process. For example the pigment inclusions found in nerve cells of old animals may be a result of this process (Fig. 5.9).

d) The autophagic vacuole or cytolysosome or autophagosome

This type of lysosome is a special case found in normal cells in which the lysosome contains a part of the cell in the process of digestion (e.g. mitochondria or portions of the ER). During starvation the liver cell shows numerous autophagic vacuoles in some of which mitochondrial remnants can be found. This is a mechanism by which the cell can achieve the degradation of its own constituents with out irreparable damage. In the liver autophagy may be induced by the injection of the pancreatic hormone glucagon (Fig. 5.9).

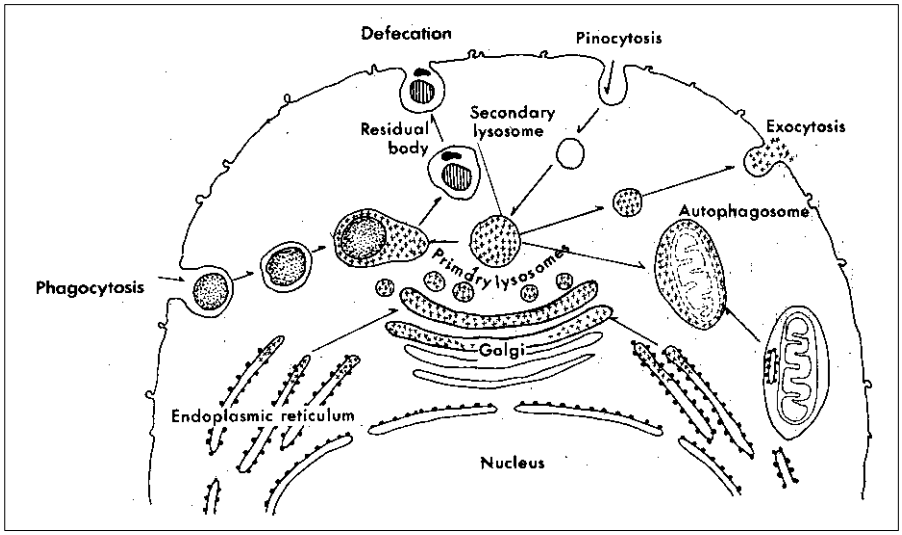

Fig. 5.9 Diagram representing the dynamic aspects of the lysosome system. Observe the relationships between the processes of phagocytosis, pinocytosis, exocytosis, and autophagy.

Origin of Lysosomes

GERL theory or Golgi Endoplasmic reticulam Lysosome theory of Novikoff

According to this theory, the products of synthetic activity of ribosomes are transferred through the smooth walled ER to Golgi body. Then, this substance is released from Golgi body in the from of vesicles which constitute the lysosomes. In some cases these are directly originated from the Golgi bodies while in few others from the endoplasmic reticulam (Fig. 5.10).

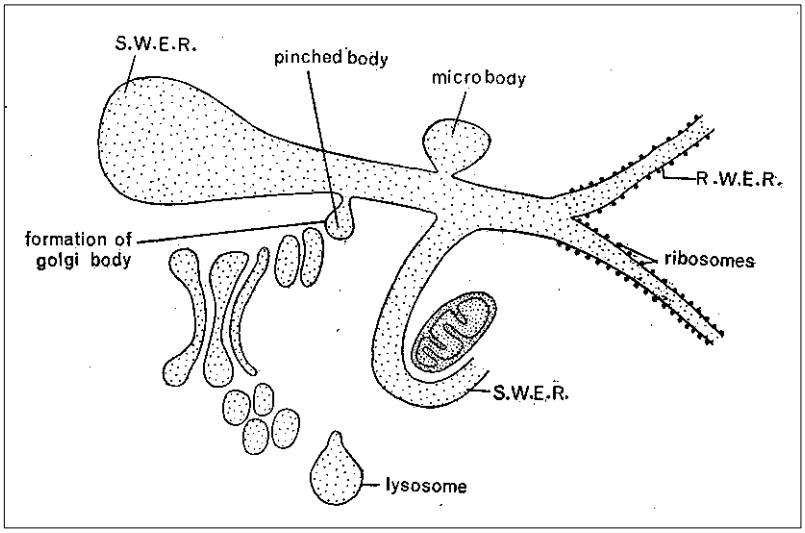

Fig 5.10 G.E.R.L. System of Novikoff

There is a strong suggestion that they have their origin from the vesicles derived from the Golgi complex (Fig. 5.11).

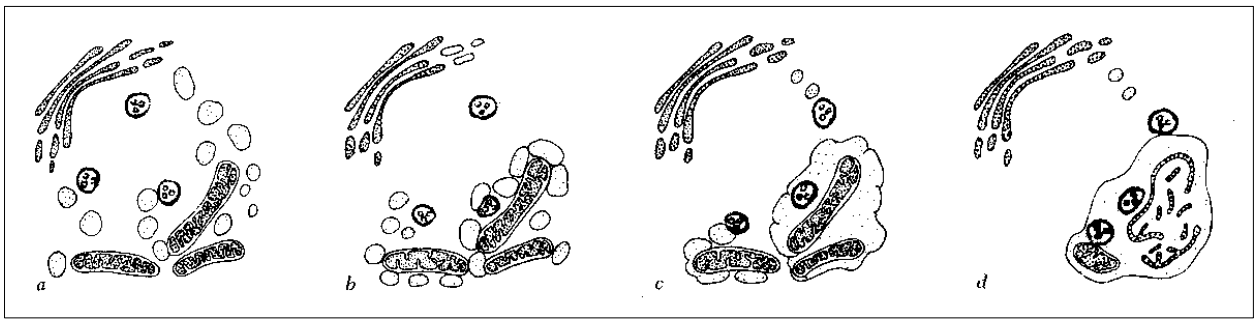

Fig 5.11 Suggested origin for lysosomes from vesicles derived from the Golgi complex. The vesicles (a) surround cellular materials, (b, c) coalesce, and then (d) digest the enclosed materials by means of hydrolytic enzymes. (Modified from Dr. D. Brandes).

Functions of Lysosomes

Lysosomes perform various functions.

a) Intracellular digestion

Lysosomal enzymes digest proteins into dipeptides and carbohydrates into monosaccharides. Some disaccharidess (sucrose) and polysaccharides (insulin and dextran) are not digested and remain in the lysosomes.

b) Autophagy

Many cellular components such as mitochondria and ER are constantly being removed from the cell by the lysosomal system. Through autophagy lysosomes are involved in renovation and turnover of cellular components. This is a mechanism by which parts of the cells are broaken down to facilitate survival.

c) Remodelling of tissues

In development lysosomes are active in remodelling of tissues (e.g.:- removal of the tad pole tail, regression of Wolffian and Mullers ducts in female and male embryos respectively and like processes).

d) Digestion of Extracellular digestion

This involves the release of primary lysosomes by exocytosis (e.g.: Osteoclasts). Osteoclasts are the multinucleotide bodies that removes bone. Degradation of bone is activated by vitamin 'A' and parathyroid hormone. In rheumatoid arthritis lysosomal enzymes erode cartilage.

e) Lysosomal enzyme involvement in Thyroid hormone release and Crinophagy

Crinophagy is a process by which excess secretory granules are removed. Thyroid hormones (Thyroxine and triidothyromin) are released from a large protein molecule (thyroglobulin) stored within the follicles. The proteolytic enzyme involved in the release is a lysosomal protease.

f) Leukocyte granules are of a lysosomal nature. These afford defence against some bacteria and virus.

g) Lysosomes are important in germ cells and fertilization. The acrosome of the spermatazoan which develops in the Golgi is a special lysosome.

h) Lysosomes are involved in Human diseases and syndromes

The study of lysosomes is particularly interesting in medicine. The lysosomes act in rheumatoid arthritis, silicosis, abestosis and gout. Lysosomess of leukocytes (specific granules) and monocytes are essential in defence against bacteria and viruses.

i) Storage diseases

These are caused by mutations that effect lysosomal enzymes. There are about 20 congenital diseases called storage diseases in which there is accumulation of substances (glycogen, glyco lipids) in lysosomes. These diseases are due to the lack of certain lysosomal enzymes. E.g.:- Tay Sachs disease. Tay Sachs disease is an inherited recessive disorder that results in mental retardation, derangement of central nervous system and death by the age of 5. In normal people the ganglioside GM_2, a constituent of plasma membranes of mammulation cells (nerve cells in particular) is continually synthesized and degraded. The membranes of the brain cells in Tay Sachs victims accumulate GM_2 due to the absence of a specific lysosomal hydralase β-N hexosaminadase A, a key enzyme in normal turnover of GM_2. The excess GM_2 is believed to cause all of the symptoms of the Tay-sachs disease.

j) Lysosomes are found in certain plant cells and they have a role in seed germination. In seedlings they are involved in hydrolysis and removal of protein and starch during germination.

4.3 SUMMARY

Chloroplasts are the most important plastids that contain the green pigment chlorophyll. They are discoid ranging in size from 4-6 μm. Electron microscope reveals their three main components the double membraned envelope, stroma and thylakoids. Chloroplast has ribosomes and DNA. Photosynthesis is the function of chloroplasts. They trap light energy and transform it into chemical energy like mitochondria, chloroplasts exhibit a certain degree of functional autonomy. They originate from proplastids by division. The symbiont hypothesis is also applied to chloroplasts. They are the dual energy converters since the energy of sugars and A T P can be used by the cell in various ways.

Lysosomes which contain the lytic enzymes exhibit polymorphism with regard to their size and chemical content. They are classified into primary and secondary lysosomes. They perform several functions of which extracellular and intra cellular digestion seems to be their main function. In conclusion we can say that they are the disposal units of the cell, removing the foreign bodies and elements of cellular architecture no longer needed. They also seem to increase in cells destained to breakdown and die. Cell death and replacement are part of the normal developmental processes and lysosomes provide destructive enzymes needed for the dissolution of such cells. Presumably the containment of these enzymes within a lysosome provides the means of selective destruction. Rupture of the membrane release these into the body of the cell and cytolysis occurs.

4.4 QUESTIONS

1. Write about the ultra structure and function of chloroplast

2. Write about the morphology and functions of lysosomes.

3. Write notes on:
    a) Thylakoids
    b) Symbiont hypothesis
    c) Genome of chloroplast
    d) Polymorphism of lysosomes
    e) Storage diseases
    f) Origin of lysosomes

4.5 REFERENCE BOOKS

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Prof. N. Lakshmi

M.Sc. BOTANY (Final)

Unit-II

Lesson No.1

1. M I C R O S C O P Y

1.0 OBJECTIVE

In this chapter resolving Power, Principles and light pathways of various light microscopes and electron microscope have been discussed.

1.1 INTRODUCTION

Although the use of lenses for magnification has been known for centuries, modern microscopy began when a Holland's eyeglass maker, Z. Jannsen, lined up two lenses to effectively multiply their individual magnifications. Subsequently, Robert Hook (1665) and Leeuwenhoek (1674) published the initial microscopic examinations of biological tissues.

A microscope can be compared with a human eye, since both have lens systems and in both cases images of the objects are formed. The most important principle involved in microscopy is to get a magnified image, in which structures may be resolved which otherwise cannot be resolved with the unaided eye.

1.1.1 Resolving Power

The ability of lens system to distinguish two adjacent points as distinct and separate or, in other words, the ability of microscope to produce separate images of closely placed objects is

known as resolving power. This depends upon the wavelength of light and numerical aperture (NA).

The Swiss physicist E. Abbe developed the theory of microscope resolution and expressed the Resolution Limit (RL) in the following formula:

$$RL = \frac{0.61\,\lambda}{NA}$$

Where λ = Wavelength of light [400-750 nm for visible light used in compound microscope; blue light (400 nm) is better for resolution than red light (700 nm)].

NA = n sin ϕ (Numerical aperture)

n = Refractive index of the medium present between the specimen and lens. Generally immersion oil (*n* = 1.5) is better than air (*n* = 1.0) (Table 1).

ϕ = Half of the angular width of the cone by the objective lens from the typical point of the specimen (Fig. 1).

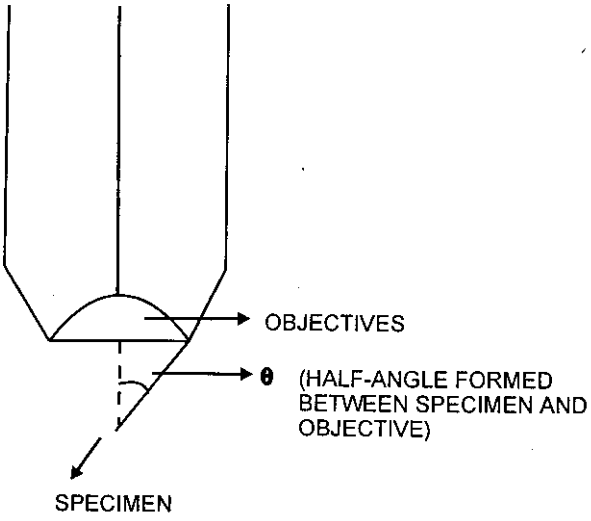

Fig. 1 Determination of Angle θ

Table 1 Refractive Indices of Some Mounting Media

Medium	**Refractive index (*n*)**
Distilled water	1.33
Glycerol	1.47
Cedar wood (immersion oil)	1.51
Eucalyptus oil	1.46
Carbon tetrachloride	1.46
Olive oil	1.47
* Euparal	1.48
Sandalwood oil	1.51
* Canada balsam	1.54
* Polystyrene	1.59

* Permanent mounting media

So numerical aperture is nothing but the light collecting ability of lens system. The higher the numerical aperture, the higher will be the degree of resolution whereas the lower the wavelength of the light, the higher will be the resolution.

Clearly, lowering RL can be achieved in three ways: (1) lowering the wavelength (λ), (2) raising the refractive index, and (3) raising sin θ as lenses of shorter focal length are used, the distance between the object and the lens decreases (Table 2).

*Table 2. Optical Properties of Objective Lenses

Magnification	Focal length (mm)	Lens-specimen distance (mm)	NA
10	16	5.5	0.25
40	4	0.6	0.65
95	2	0.1	1.32

*Adopted from Alberts *et al*. (1994).

If λ = 400 nm, n = 1.5, sin θ = 0.99 and an optical lens of maximal NA, it gives a theoretical maximum resolution of the objective lens of about 0.2 μm which is 1000 times greater than that of unaided human eye.

A wide variety of microscopes are currently available for studying plant structure. These are mainly categorized into two: (i) Light Microscope, and (ii) Electron Microscope. In the former one, magnification is achieved with the help of optical lens system. Brightfield, darkfield, phase-contrast, polarization and fluorescence microscopes come under this category. Whereas in the latter category a beam of electrons is used in place of light to obtain higher magnification; transmission and scanning electron microscopes belong to this category.

The resolution of various biological components is given as follows (adopted from Lewis and John, 1963):

Structure	Atoms, molecules	Macro-mole-cules	Genes, viruses	Bacteria, cytoplasmic organelles	Chromo-somes, cells	Tissues
Scale	1 A^o	10 A^o	100 A^o 1000 A^o	1 μ	10 μ	100 μ
Means of Resolution	←x-ray diffraction→		←Electron microscope→		←Light microscope→	

1.2 LIGHT MICROSCOPY

1.2.1 Bright-Field Microscope

Bright-field microscope is absolutely indispensable to the biologists. It provides magnification of the objects which are not visible to naked eye. In this commonly used compound microscope, dark image of the object appears against bright background. This is accomplished by the condenser, a series of lenses and diaphragms below the stage on which the object is located.

Lens systems

Compound microscope consists of three lens systems: (1) objective, (2) eyepiece or ocular, and (3) condenser (Figs. 2, 3).

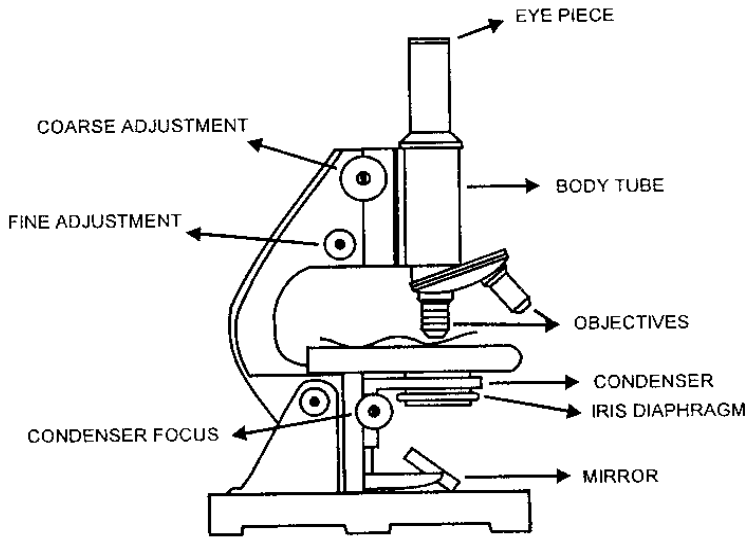

Fig. 2 The Compound Microscope

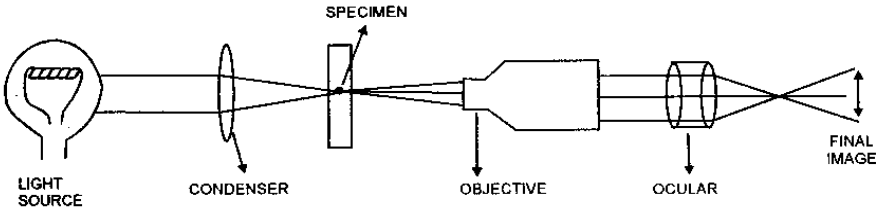

Fig. 3 The Path of Light through Bright-field Microscope

(1) **Objective:** Objective is considered to be the most important component of the light microscope since it affects the quality of image formation. On the basis of degree of correction for chromatic aberration, objectives are of two types: (i) achromatic objectives that are corrected for two regions of the spectrum, and (ii) apochromatic objectives that are corrected for three regions of the spectrum (for details see Desai and Desai, 1980; O'Brien and McCully, 1981).

Light focused on the specimen is either differentially transmitted (absorbed) or, for the background, fully transmitted to the objective. This lens resolves the specimen to produce a magnified image and focus in the microscope tube. Generally, compound microscopes are fitted with three objectives with different magnifying powers. They are low power, high power and oil-immersion objectives. These are easily recognised by their length and NA value engraved on the barrel. Low power objective is the shortest and oil-immersion is the longest.

In bright-field microscope, both direct (rays not hitting the specimen) and indirect rays (rays diffracted or scattered from specimen) enter the objective, hence dark image is seen against bright background.

(2) Eyepiece (ocular): The main purpose of ocular is to magnify the intermediate image and to correct certain aberrations produced by the objective. Ocular is composed of mainly two lenses; the upper component or eye lens is the magnifier, whereas the lower component is known as field lens.

(3) Condenser : The quality and method of use of condenser are important factors for a good microscopy. The primary function of condenser is to supply sufficient cone of light to the objective to gain the maximum resolution. So, it should be properly positioned during the microscopy. The Abbe condenser, aplanatic condenser and achromatic condensers are available for general purposes. Condensers are also fitted with iris diaphragm and filter holder to control the light intensity.

All the four optical components (lamp, condenser, objectives and eyepiece) are installed in the common axis in the microscope. The following steps should be carefully taken for the effective use of research microscope (see also Desai and Desai, 1980; O'Brien and McCully, 1981):

1. Turn on the lamp, adjust it to a considerable intensity.

2. Adjust the mirror in such a way that the light travels up the body tube of microscope.

3. Take out the eyepiece and examine the back focal plane of the objective. Close the condenser iris until about ¾ of the field of vision is left clear. In some binocular microscopes, it is difficult to see the image of the condenser iris in the back focal plane of the objective. In this case, close the iris, then open it slowly and watch the ‘Circle of light’ as it increases. It comes to a certain position after which further increase in iris diameter does not increase the ‘Circle of light’. Close down the iris a little from that position.

4. Place the specimen on the stage.

5. Now keep the low power objective in position. Lower the body tube with the help of coarse adjustment knob until the distance between the specimen and low power objective becomes just about 5-7 mm.

6. Bring the specimen in focus with the help of coarse adjustment knob by looking through the eyepiece.

7. Sharpen the image with fine adjustment knob.

8. When higher magnification is needed, change the objective by shifting the nosepiece in the appropriate direction till it clicks into place. Care should be taken not to touch the objective lens with our fingers.

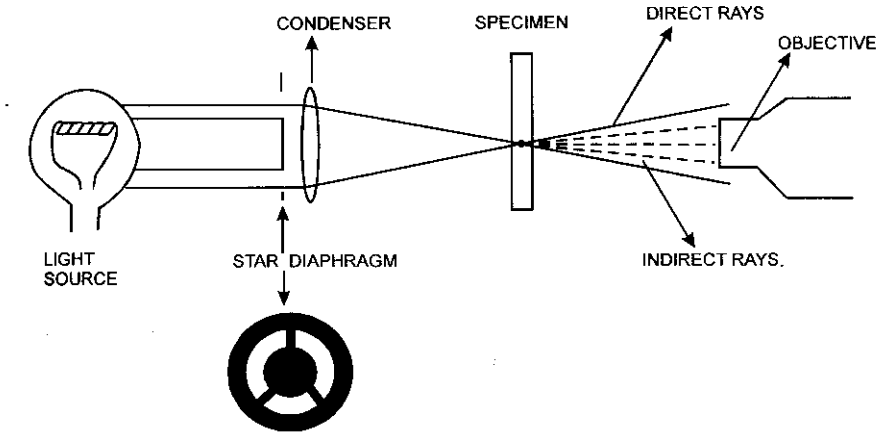

Fig. 4 Dark-field Microscope

9. Focus the specimen again with the help of coarse and final adjustments. Adjustment of iris is necessary again since different power objectives have different field areas.

10. To set up oil-immersion objective, especially x90 or x100, first rack the body tube up and place a drop of immersion oil (free of bubbles).

11. By observing from the sides of the instrument, rack the objective down to touch the oil and almost near to the specimen. Care should be taken to see that the objective does not touch the specimen.

12. By looking through the eyepiece, slowly focus upwards with the fine adjustment until clear image appears. It comes quickly, since the working distance of oil-immersion objective is about 0.1-0.15 mm.

13. Remove and clean the oil from both objective lens and slide with lens paper immediately after the use of instrument

1.2.2 Dark-Field Microscope

In dark-field microscope, bright image of specimen is seen against the dark background. A specially devised condenser with star diaphragm is used in this microscope. It blocks the light rays (direct rays) that would normally enter the objective lens. Instead, it supplies a hollow cone of light and illuminates the specimen. Only indirect rays scattered from specimen would enter the objective. As a result bright image against the dark background is seen (Fig. 4). In this microscope, special condensers of very high NA, such as cardoid and the paraboloid, are employed with oil-immersion objective.

Since the contrast is quite vivid (just like stars against a dark sky) organelles such as mitochondria and lysosomes are easily detected by this microscope. It is also useful for the observation of plant structures and microbes in an unstained condition. It has a diagnostic significance especially in the case of syphilis (Desai and Desai, 1980).

1.2.3 Phase-Contrast Microscope

Phase-contrast microscope is based on phase-contrast principles propounded by Fritz Zernike. He got the Nobel Prize in Physics in 1953 for his phase-contrast principles. According to him, light waves have variable character for frequency and amplitude. Human eye cannot notice a phenomenon when two light rays have similar amplitude and frequency, but different phase (Fig. 5). This can be achieved by phase-contrast microscope. When the light rays are passing

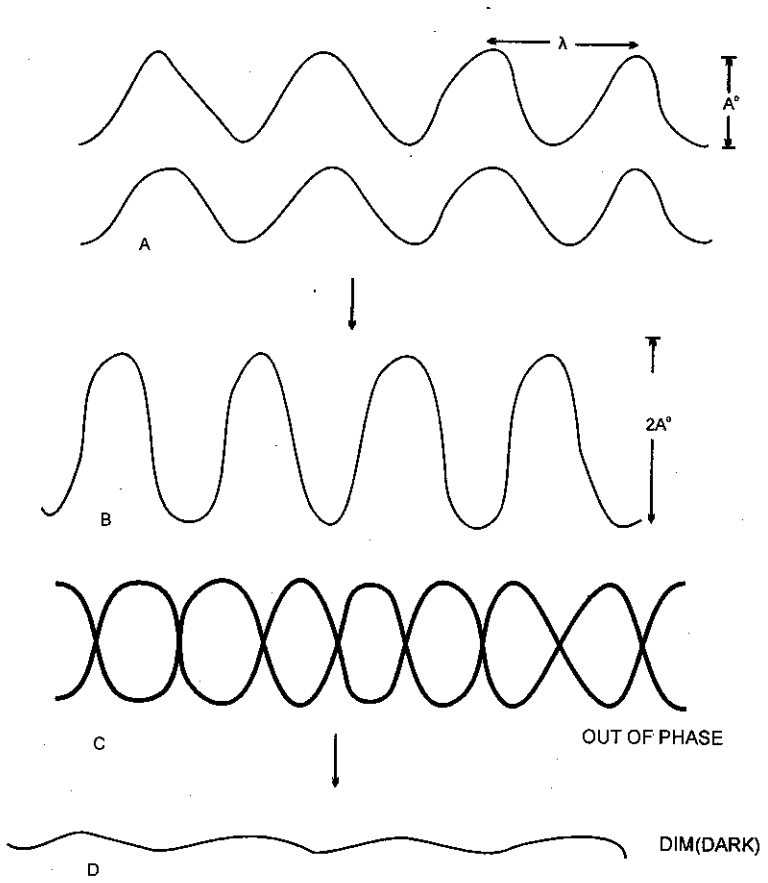

Fig. 5. Properties of Light

(a) Both rays are in phase (rays go up at the same time and down at the same time), (b) Constructive interference in which amplitude becomes doubled ($2A^o$), brightness increased, (c) Rays are in out of phase, and (d) Destructive interference, rays that are out of phase partially cancelled, brightness decreased and finally becomes dark.

through the vacuum, they travel in high speed. When they pass through the transparent cells, light rays become slow due to change in the velocity. When a beam of light changes speed, it is refracted at an angle depending on the magnitude of the velocity change. So the light rays are refracted and alter their phase. This phase change may not be noticed by the human eye. In the phase-contrast microscope, both refracted rays (indirect rays) and direct rays are undergoing interference. As a result clear image of the specimen is seen.

Phase-contrast microscope is fitted with two additional optical lenses, namely annular diaphragm and phase-shifting plate (Fig. 6). Annular diaphragm is attached in place of iris diaphragm to the condenser. This allows only a ring of light to pass through the condenser and then to the specimen. Each objective requires different size of annular diaphragm according to its numerical aperture. Phase-shifting plate is attached at the back of the objective. It involves phase-shifting of direct rays with respect to indirect rays.' Phase-shifting plate is composed of a disk of glass having circular trough etched in it and when light passes through it, it creates a phase difference of a quarter of a wavelength as compared with the rest of the plate. It enhances the difference between indirect and direct rays so that interference takes place and clear image of the object is seen.

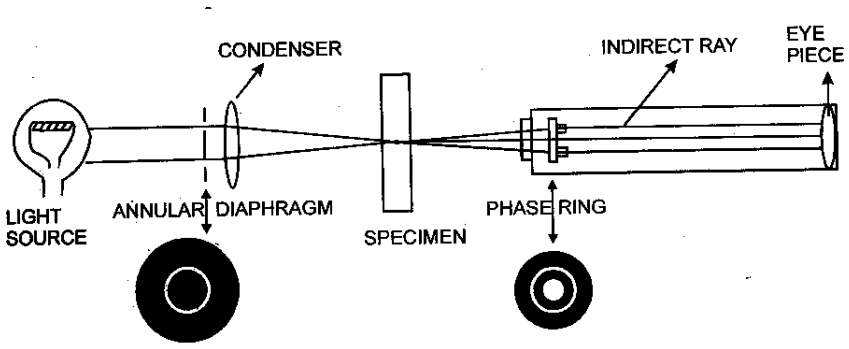

Fig. 6 Phase Contrast Microscope

Depending on the type of phase-shifting element employed, the specimen appears darker against light background (positive contrast) or lighter against dark background (negative contrast) (see also O'Brien and McCully, 1981). Phase-contrast microscope is widely used to observe living cells and organelles.

1.2.4 Polarization Microscope

Polarization microscope is used to visualize birefringents. Various biological structures like microtubules, micro fibrils and crystals show birefringence property because they refract polarized light in different ways depending on the direction from which the light strikes the specimen. Anisotropic materials are briefringents, because they show double refractions. The principle of the polarizing microscope is to deduce and measure the structural anisotropy of the specimen from the

optical anisotropy- that it displays (see also Bennett, 1961; Bartels, 1966; James, 1976; O'Brien and McCully, 1981).

When a beam of polarised light passes through the birefringent object, the ray splits into two rays polarised in mutually perpendicular lines; the one which follows the ordinary laws of refraction is known as the 'ordinary' ray and the other, whose velocity through the object is different, is known as the 'extraordinary' ray. The difference in refractive indices *(n_e - n_o)* is the value of birefringence (B). The two polarised rays, after emerging from the object, recombine but, because of different velocities through the object, one shows retardation when compared to the other. The value of 'retardation' (T), which is based on the birefringent property, is measured in the following manner:

$T = B \times t$

where T = Value of retardation
B = birefringence, i.e., $(n_e - n_o)$ and
t = thickness of the object

Finally $T = (n_e - n_o)\ t$; it is expressed in terms of wavelength in Angström units (Sharma and Sharma, 1972).

Polarization microscope is fitted with two additional optical lenses: (1) a vertical polarizer placed between the light source and substage condenser to produce polarized light, and (2) an analyzer horizontally placed above the objective lens (Fig. 7). In this, a bright image of specimen against dark background appears. Polarizing microscope is also useful for studying the optical properties of cell wall and starch grains.

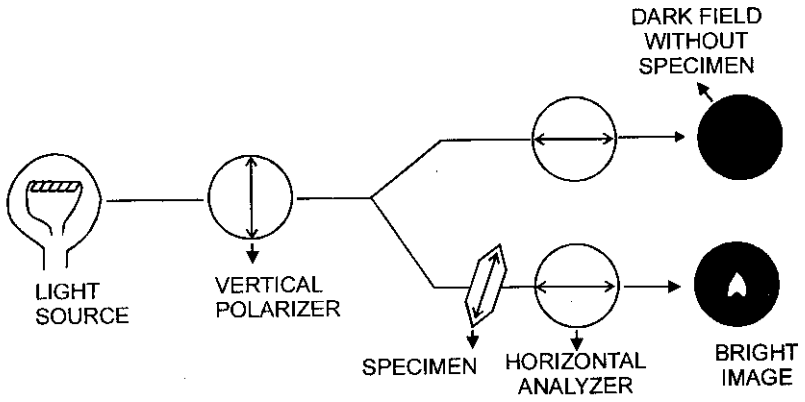

Fig. 7 Polarization Microscope

1.2.5 Fluorescence Microscope

Fluorescence microscope is based on the principle 'Fluorescence'. In this microscope, dyes are used that absorb light energy of one wavelength and emit light energy of longer wavelength; for example, the dye fluorescein absorbs at 490 nm and emits at 520 nm appearing yellow green and

rhodamine absorbs at 546 nm and emits at 580 nm appearing red. So each compound has a characteristic absorption and emission spectrum (see also Pringsheim, 1963; Udenfriend, 1964, 1969).

A fluorescence microscope is fitted with two filters, these are (1) a *primary* or *exciter filter* placed between the light source and specimen, and it allows only the excitation wavelength, and (2) a *secondary* or *barrier filter* placed between the specimen and viewer, and it ideally transmits only the wavelengths emitted by the fluorescing compounds (Fig. 8).

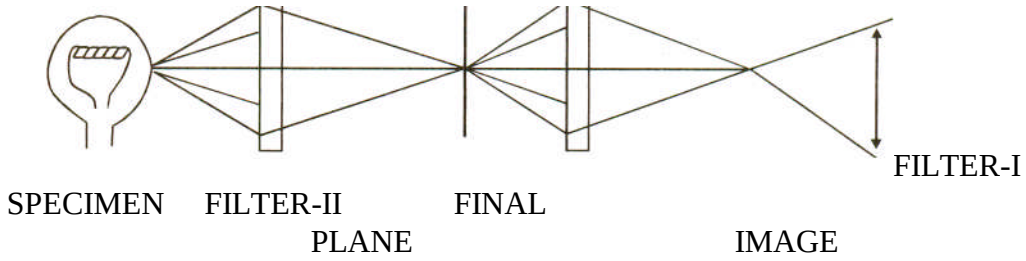

Fig. 8 Fluorescence Microscope

Fluorescence in histological specimens is induced either in compounds inherent in the tissue known as autofluorescence or in compounds (fluorochromes) that have been added to the tissue. Freehand sections, peels and whole mounts are enough to study the autofluorescence of lignins and chlorophylls present in higher plants. Plant cuticles (van Gijzel, 1975) and suberized walls of cork (Mader, 1954) are also auto fluorescent. The compounds of biological origin like berberin sulphate (from *Berberis)* and Primulin *(Primula)* and other compounds are still widely used as fluorochromes. Peirson and Dumbroff (1969) used an extract from *Chelidonium* roots to show effectively the casparian strips in roots. The other fluorochromes are acridine orange for DNA (Bertalanffy, 1963; Pearse 1972; Kasten, 1967), ethidium bromide for DNA and lignified walls, and aniline blue for callose of sieve elements (Currier and Strugger, 1956; Eschrich and Currier, 1964; Smith and McCully, 1978b; O'Brien and McCully, 1981).

The major use of fluorescence microscope in microbiology is in immuno-fluorescence studies. The antibody is made fluorescent by conjugating it with a fluorescent dye. With the help of fluorescence microscope, it is possible to detect specific types of antigens using an antibody tagged with a fluorescent dye (see also Nairn, 1969; Walker *et al*., 1971; Desai and Desai, 1980).

1.3 ELECTRON MICROSCOPY

In electron microscope instead of light a high speed electron beam is used (Table 3). Visualization of minute structures like ribosomes, mitochondria and membranes is possible with this microscope. In 1924, F. deBrogile (quoted in Prescot *et al*., 1999) opined that electrons could

behave as waves with the wavelength inversely proportional to the square root of the voltage used to generate the electrons.

$$\lambda = 12.3 / [(\sqrt{V})\ (0.1)]\ nm$$

Table 3. Characteristic differences between electron and light microscopes

Sl. No.		Electron microscope	Light microscope
1	Best resolution	0.0005 μm (0.5 nm)	0.2 μm
2	High vacuum	Necessary	Not necessary
3	Specimen	Dead; ultrathin sections	Living and non-living material; sections of 2-10 μm thickness
4	Radiation source	Electron beam	Visible light
5	Lenses	Electromagnetic lenses	Glass lenses
6	Source of contrast	Scattering of electrons	Differential light absorption
7	Staining	Heavy metals	Dyes
8	Focussing mechanism	Adjacent current to the magnetic lens	Adjacent lens position
9	Specimen mount	Metal grid (copper)	Glass slide

1.3.1 Transmission Electron Microscope (TEM)

In TEM, the commonly used voltage is 100,000, so the wavelength of the electrons is 0.004 nm. Aberrations inherent in the lenses of the instrument require its operation at very low numerical apertures (0.001). So the resolution limit, according to Abbe's formula, is approximately 0.2 nm. Compared to the unaided human eye (200 μm or 200,000 nm), this represents the magnification upto 1,00,000 X (see also Alberts *et al*., 1994).

Although the source of illumination is electron beam and not light, in general, the TEM is quite similar to the bright-field light microscope (Fig. 9). There are some differences between these two (Table 3). In TEM, high speed electron beam generated from electron gun is used. Electrons are focussed by electromagnetic lenses. Since air molecules interfere with the movement of electrons, high vacuum is created within the microscope. Also due to very poor penetrating capacity of electrons, sections should be ultrathin. These conditions do not allow the observation of living material through electron microscope.

There are three electromagnetic lenses in electron microscope. The condenser lens, placed between the source of illumination and specimen, collimates the electron beam on the specimen and an enlarged image is produced by two other lenses similar to the objective and ocular of light microscope. Since the electrons are not visible to the human eye, the final image is projected on the fluorescent screen.

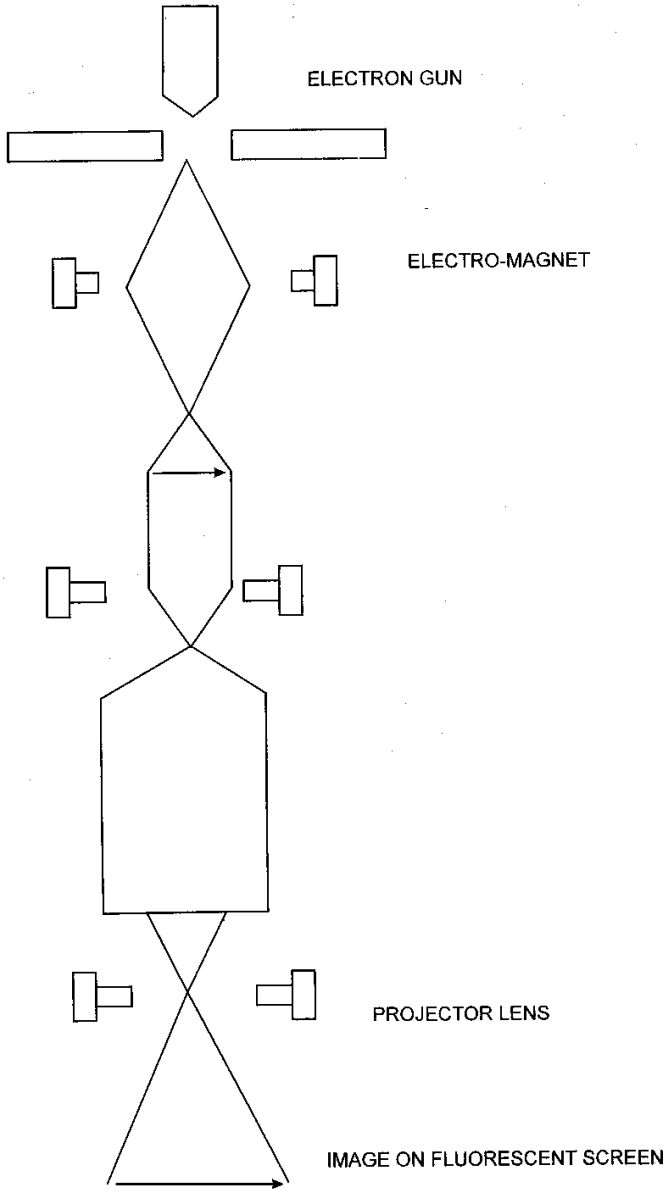

Fig. 9 Transmission Electron Microscope

Since most of the constituent elements in biological matter are of low mass, the contrast of these materials is very poor. This can be enhanced by staining with salts of heavy metals like uranyl acetate, lead citrate etc. These metals may be either fixed on the specimen (positive staining) or used to increase the opacity of the surrounding area (negative staining). The latter is useful 'for observation of virus particles and bacteria.

1.3.2 Scanning Electron Microscope (SEM)

The electron microscope has been modified to provide three dimensional image of the specimen. The specimen is surface coated with a thin layer of heavy metal and is then scanned by a narrow electron beam. The intensity of various radiations released from a portion of the specimen depends on the shape and chemical composition of the irradiated object. Electrons scattered or emitted from the specimen's surface will form the image. The final image can be viewed from the fluorescent screen. So the topology of the object can be studied with SEM.

SEM lacks high resolution capacity when compared to TEM but it has the advantage of revealing three-dimensional impression. With this microscope, the surface features of the specimen can be studied with a high contrast.

Some of the important features of the microscopes are given in Table 4.

Table 4. Characteristic Features of Some Important Microscopes

Type of microscope	Image formation	Magnification	Applications
Bright-field microscope	Dark image of the object against bright background	1000-2000 x	To study the anatomy, embryology of plants and gross morphology of yeast, moulds, algae, protozoa, bacteria etc.
Dark-field microscope	Bright image of the object against dark background	1000-2000 x	To study the gross morphology of the living specimen
Phase contrast microscope	Based on phase contrast principles; Darker against the light background (positive contrast) or lighter against the dark background (negative contrast)	-do-	To study the gross morphology of living specimen and cell organelles.
Polarization microscope	Bright image of the object against dark background	-do-	To study the birefringents, viz., microtubules, microfibrils, crystals and also to study the optical properties of cell wall.
Fluorescent microscope	Based on fluorescent principle	-do-	To study the plant cuticles, suberized walls, lignins, chlorophylls, DNA, callose of sieve elements; immuno-fluorescence studies in microbiology
Electron microscope	Viewed on fluorescent screen	Upto 10,00,000 x	To study the ultrastructure of cell organells, microbial cells, viruses etc.

1.4 SUMMARY

Resolving Power

The ability of microscope to produce separate images of closely placed objects is known as resolving power. This depends upon the wavelength of light and numerical aperture (NA). This can be expressed in the form of *Resolution Limit* (RL) in the following formula:

$$RL = \frac{0.61\ \lambda}{NA}$$

Light Microscopy

Bright-field microscope is commonly used compound microscope, in which dark image of the object appears against bright background. In general, compound microscope consists of three lens systems: (1) Objective, (2) Eyepiece or ocular, and (3) Condenser. The objective lens resolves the specimen to produce a magnified image and focus in the microscope tube. The ocular is to magnify the intermediate image and to correct certain aberrations produced by the objective. The primary function of condenser is to supply sufficient cone of light to the objective to gain the maximum resolution. In *Dark-fied microscope*, bright image of specimen is seen against the dark background. *Phase-contrast microscope* is based on phase-contrast principles propounded by Fritz Zernike. In this microscope, both refracted rays (indirect rays) and direct rays are undergoing interference. As a result, clear image of the specimen is seen. *Polarization microscope* is based on the birefringence property of various biological structures like microtubules, microfibrils and crystals. The principle of the polarizing microscope is to deduce and measure the structural anisotropy of the specimen from the optical anisotropy that it displays. *Fluorescence microscope* is based on the principle 'Fluorescence'. In this microscope, dyes are used that absorb light energy of one wavelength and emit light energy of larger wavelength. Biological specimens are stained with dyes and observed under this microscope.

Electron Microscopy

In *Transmission electron microscope*, high speed electron beam is used. Visualization of minute cell organelles like ribosomes, mitochondria and biological membranes is possible with this microscope. Electrons are focussed onto the specimen by the electromagnetic lenses. Since most of the constituent elements in biological matter are of low mass, the contrast of these materials is very poor. This can be enhanced by staining with salts of heavy metals like uranyl acetate, lead citrate etc., *Scanning electron microscope* provides three dimensional image of the specimen. With this microscope, the surface features of the specimen can be studied.

MODEL QUESTIONS

Essay Questions

1. Define resolving power. Discuss the principles and light pathways of various light microscopes with special reference to phase-contrast and polarization microscopes.

2. Write an essay on Transmission Electron Microscope and discuss the characteristic differences between electron and light microscopy.

Short Notes

1. Resolving power of the microscope
2. Compound microscope
3. Phase-contrast microscope
4. Polarization microscope
5. Fluorescent microscope
6. Scanning electron microscope

REFERENCE BOOKS

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2. Khasim, S.M. 2002. Botanical Microtechnique: Principles and Practice. Capital Publishing Company, New Delhi.

Dr. S.M. KHASIM

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M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-II

Lesson 2

Cell signaling and signal transduction

2.0 **OBJECTIVE:**

The chapter deals with the overall view of the cell signalling mechanism, the major components that perceive and transmit signals and about signal delivery.

3.1 INTRODUCTION

Cells respond to their environment by reorganizing their structure, regulating the activity of proteins and altering patterns of gene expression. The stimulus for such response is termed a signal. The external stimuli may be a small molecule, a macro molecule or a physical agent, such as light. The passage or transfer of such signals to the receptor sites of the cell is called signal transduction. Signals interact with the responding cells through molecules termed receptors.

Cells of all organisms have evolved mechanisms to sense the changes in the environmental stimuli (both external and internal) such as presence of growth factors, a new chemical like antibiotic, hormones or physical factors like temperature, light, odour or even microbes etc., through certain signals. Common examples for such changes in plants include increase in temperature leading to the production of ‘heat shock proteins’, production of chlorophyll in germinating seedlings only after exposure to light etc. Depending on the nature of signal and the response of the cell or organism, certain genes may be modulated for their expression – some genes

may be silenced (inactivated or switched off) or induced into activity ('switched on') or the level of activity/function may be quantitatively regulated).

Small molecules often act as diffusible signals. In unicellular organisms, diffusible signals may be environmental in origin or may be released from other cells. Signals may be released from nearby cells and diffuse over short distances. In animals based on the distance over which the signal acts, they are classified into three types. A molecule that selectively binds to a receptor is termed a ligand. Some well understood cell surface receptor proteins involved in signal transmission in Eukaryotes are given below:

Table 1 Cell surface receptor proteins involved in signal transmission into eukaryotic cells

Receptor type	Description	Signals
G-protein-coupled receptors	Activate intracellular G-proteins, which bind GTP and control biochemical activities by conversion of this GTP to GDP with the release of energy	Diverse: epinephrine, peptides (e.g. glucagon), protein hormones, odorants, light
Tyrosine kinases	Activate intracellular proteins by tyrosine phosphorylation	Hormones (e.g. insulin), various growth factors
Tyrosine kinase associated	Similar to tyrosine kinase receptors but activate intracellular proteins indirectly (e.g. see description of STATs in the text)	Hormones, growth factors
Serine-threonine kinases	Activate intracellular proteins by serine and/or threonine phosphorylation	Hormones, growth factors
Ion channels	Control intracellular activities by regulating the movement of ions and other small molecules into and out of cells	Chemical stimuli (e.g. glutamate), electrical charges

To exert an effect on genome activity, the nutrient, hormone, growth factor or extracellular compound must influence events within the cell by two ways (Fig. 2.1).

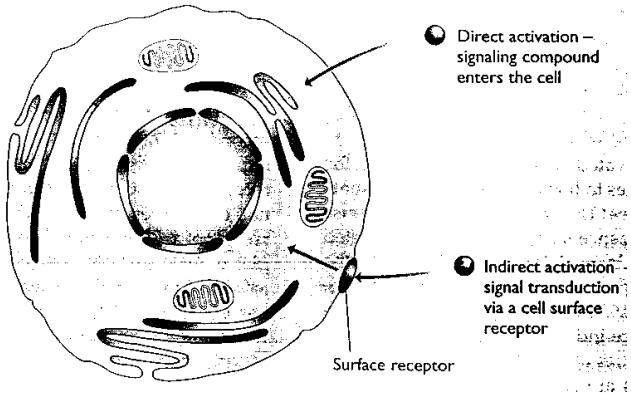

Fig. 2.1 Two ways in which an extracellular signalling compound can influence events occurring within a cell

1. Directly by acting as a signaling compound that is transported across the cell membrane and into the cell (Fig. 2.1).

2. Indirectly by binding to a cell surface receptor which transmits a signal into the cell (Fig. 2.1). Signal transmission is one of the major research areas in cell biology. We study in brief about a few signaling compounds and their significance in signal transduction pathway.

Endocrine signalling – Hormones act on target cells distant from the site of synthesis by cells of endocrine organs e.g. endocrine hormone is carried by the blood (Fig. 2.2a).

Paracrine signaling – The signaling molecules released by a cell only affect target cells in close proximity to it e.g. conduction f an electric impulse from one nerve cell to another or from a nerve cell to a muscle cell (Fig. 2.2b).

Autocrine signaling – Cells respond to substances that they themselves release. Many growth factors act in this fashion. Cultured cells secrete growth factors that stimulate their own growth and proliferation e.g. tumor cells. They release growth factors that stimulate unregulated proliferation of themselves as well as adjacent non-tumor cells (Fig. 2.2c).

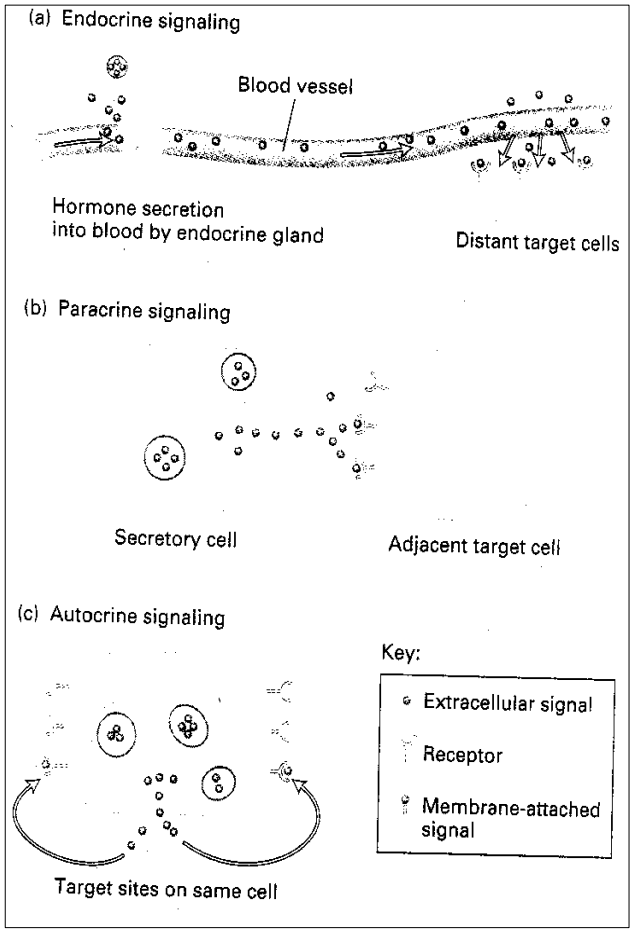

Fig. 2.2 General schemes of intercellular signalling in animals. (a-c) Cell-to-cell signalling by extracellular chemicals occurs over distances from a few micrometers in autocrine and paracrine signalling to several meters in endoctrine signalling.

2.2 SIGNAL MOLECULES – SIGNAL TRANSDUCTION

In eukaryotes, one type of cell can signal other by secreting a hormone. Hormones circulate through the body and regulate the expression of particular genes. In animals there are two classes of hormones. The first class are **steroid hormones**. They are lipid soluble molecules derived from cholestrol which play important roles in female reproductive cycles, testosterone, a hormone of male behaviour. Once they enter a cell, they interact with cytoplasmic proteins called hormone

receptors. The **receptor/hormone complex** that is formed enters the nucleus, where it acts as a transcription factor to regulate the expression of certain genes (Fig. 2.3).

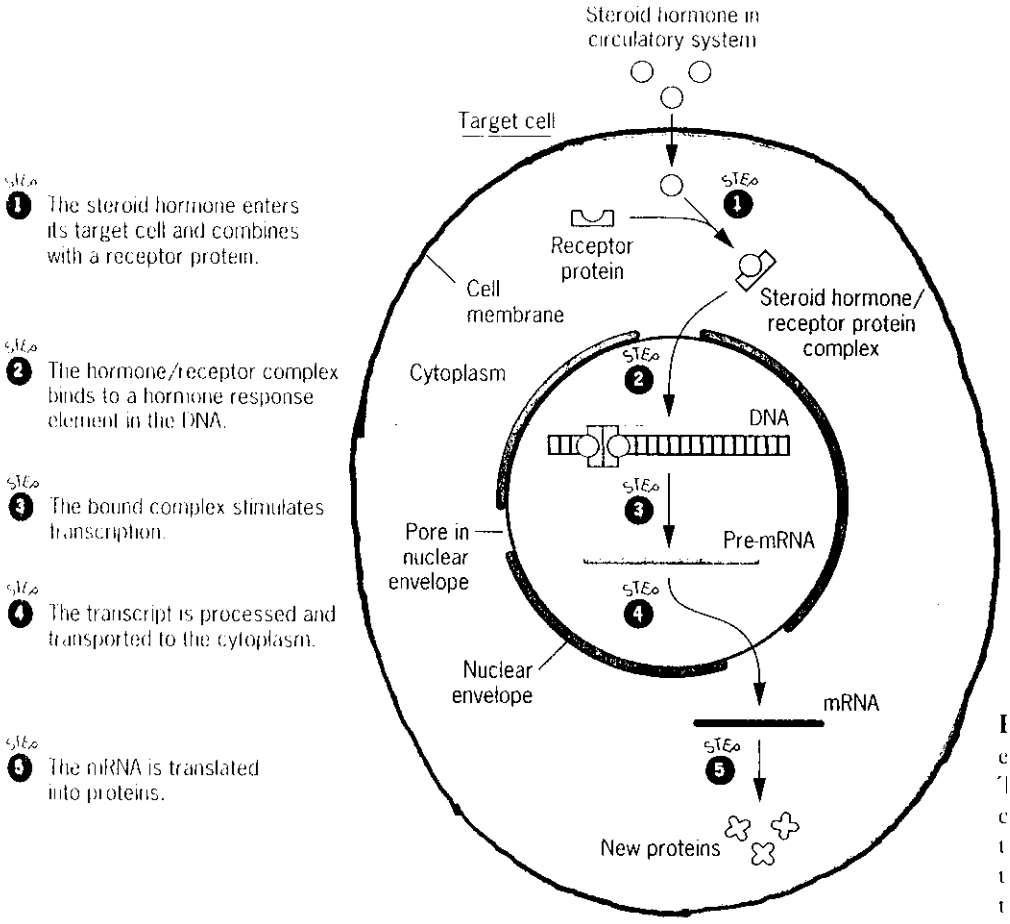

Fig. 2.3 Regulation of gene expression by steroid hormones. The hormone interacts with a receptor inside its target cell, and the resulting complex moves into the nucleus where it activates the transcription of particular genes.

The second class of hormones, the **peptide hormones** are linear chains of aminoacids. These molecules are encoded by genes, e.g. Insulin, which regulates blood sugar levels, somatostropin, a growth hormone etc. Because peptode hormone are too large to pass freely through cell membrane, the signals they convey must be transmitted to the interior of cells by membrane bound receptor proteins (Fig. 2.4). When a peptide hormone interacts with its receptor, it causes a conformational change in the receptor, leading to change in other proteins inside the cell. Through a cascade of such changes, the hormonal signal is transmitted through the cytoplasm of the cell into the nucleus, where it has the effect of regulating the expression of specific genes. This

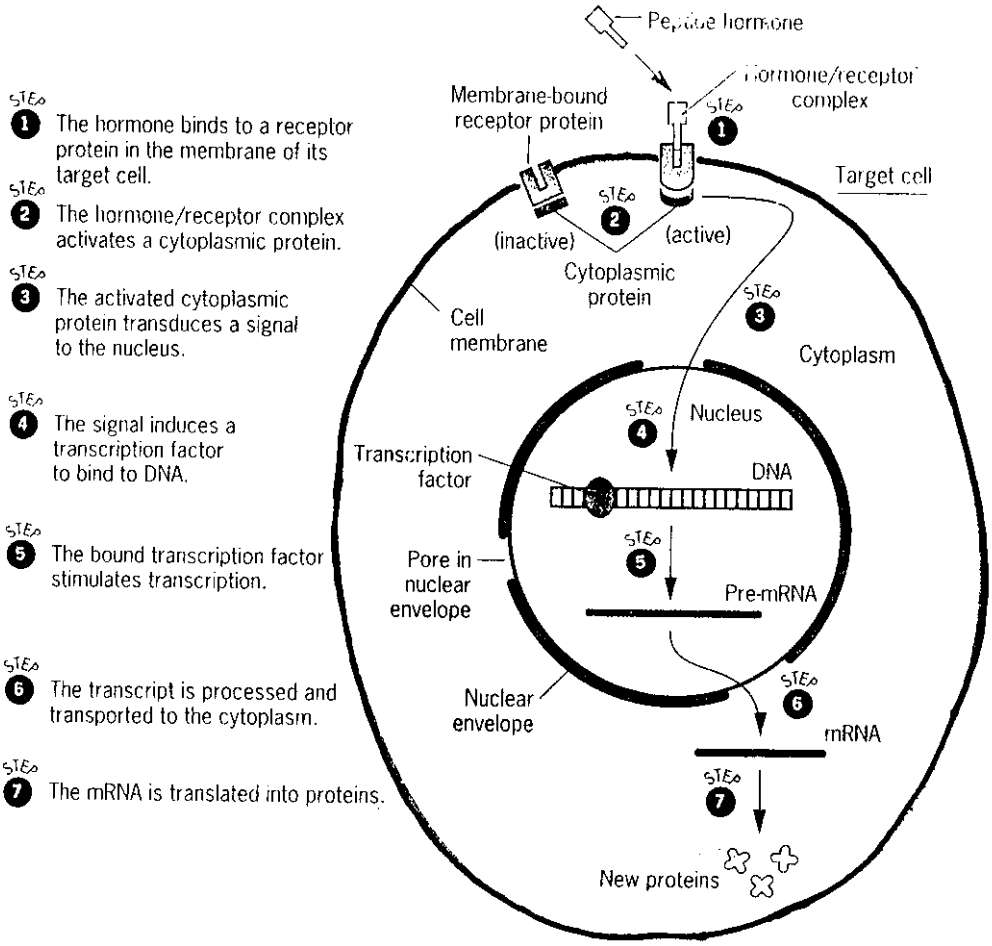

Fig. 2.4 Regulation of gene expression by peptide hormones. The hormone (an extracellular signal) interacts with a receptor in the membrane of its target cell. The resulting hormone/receptor complex activates a cytoplasmic protein, that triggers a cascade of intracellular changes. These changes transmit the signal into the nucleus, where a transcription factor stimulates the expression of particular genes.

process of transmitting the hormonal signal through the cell and into nucleus is called signal transduction.

2.2.1 Protein phosphorylation (principal mechanism for signalling)

A recurring theme in studies of signal transduction is the involvement of protein kinases. The second messengers cAMP, calcium exerts effects on cells through the action of protein kinase. Protein kinases represent critical nodes for amplification and distribution of signals, since single protein kinase can phosphorylate many different target proteins. The catalytic activities of kinases are modulated by phosphorylation. For calcium-calmodulin dependent protein kinase, the enzyme is inactive unless bound by calcium-calmodulin complex.

2.3 CELL SURFACE RECEPTORS BELONG TO FOUR MAJOR CLASSES

The different types of cell-surface receptors that interact with water-soluble ligands are schematically represented in Fig. 2.5. Binding of ligand to some of these receptors induces second-messenger formation, whereas ligand binding to others does not. For convenience, we can sort these receptors into four classes:

2.3.1 G protein-coupled receptors (Fig. 2.5a)

Ligand binding activates a **G protein**, which in turn activates or inhibits an enzyme that generates a specific second messenger or modulates an ion channel, causing a change in membrane potential. The receptors for epinephrine, serotonin, and glucagon are examples.

2.3.2 Ion-channel receptors (Fig. 2.5b)

Ligand binding changes the conformation of the receptor so that specific ion flow through it; the resultant ion movements alter the electric potential across the cell membrane. The acetylcholine receptor at the nerve-muscle junction is an example.

2.3.3 Tyrosine kinase-linked receptors (Fig. 2.5c)

These receptors lack intrinsic catalytic activity, but ligand binding stimulates formation of a dimeric receptor, which then interacts with and activates one or more cytosolic protein-tyrosine kinases. The receptors for many cytokines, the interferons,a nd human growth factor are of this type. these tyrosine kinase-linked receptors sometimes are referred to as the **cytokine-receptor superfamily**.

2.3.4 Receptors with intrinsic enzymatic activity (Fig. 2.5d)

Several types of receptors have intrinsic catalytic activity, which is activated by binding of ligand. For instance, some activated receptors catalyze conversion of GTP to cGMP; others act as protein phosphatases, removing phosphate groups from phosphotyrosine residues in substrate proteins, thereby modifying their activity. The receptors for insulin and many growth factors are ligand-triggered protein kinases; in most cases, the ligand binds as a dimer, leading to dimerization of the receptor and activation of its kinase activity. These receptors—often referred to as receptor serine/threonine kinases or **receptor tyrosine kinases**--autophosphorylate residues in their own cytosolic domain and also can phosphorylate various substrate proteins.

Most receptors for extracellualr signals are on the cell surface because the signalling molecules are hydrophilic and unable to penetrate the plasma membrane. Other molecules can cross the plasma membrane directly and interact with cytoplasmic or nuclear receptors, and these often initiate simple signal transduction pathways.

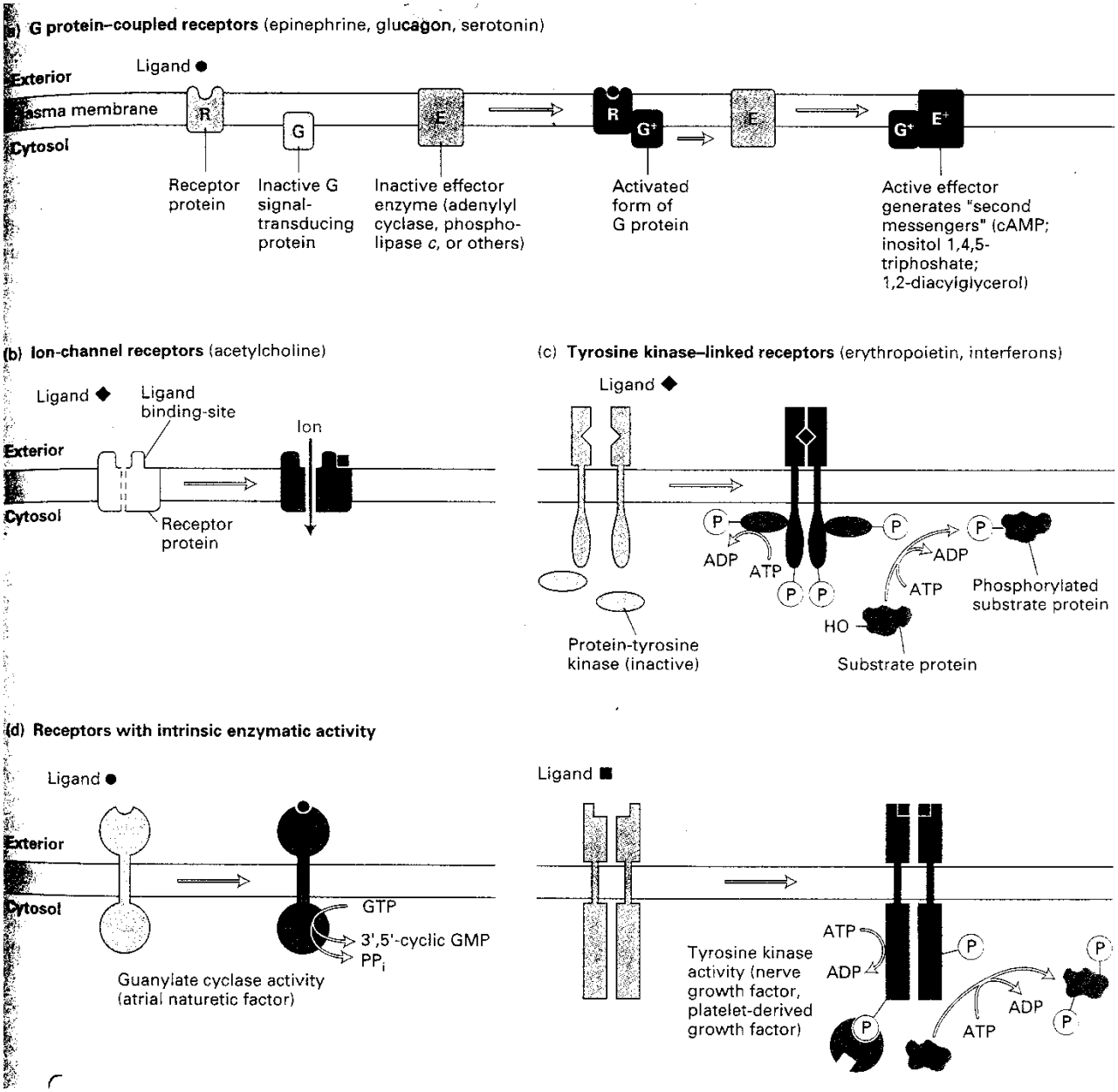

Fig. 2.5 Four classes of ligand-triggered cell-surface receptors. Common ligands for each receptor type are listed in parentheses. (a) g protein-linked receptors. Binding of ligand (maroon) triggers activation of a G protein, which then binds to and activates an enzyme that catalyzes synthesis of a specific second messenger. (b) Ion-channel receptors. A conformational change triggered by ligand binding opens the channel for ion flow. (c) Tyrosine kinase—linked receptors. Ligand binding causes formation of a homodimer or heterodimer, triggering the binding and activation of a cytosolic protein-tyrosine kinase. The activated kinase phosphorylates tyrosines in the receptor; substrate proteins then bind to these phosphotyrosine residues and are phosphorylated. (d) Receptors with intrinsic ligand-triggered enzymatic activity in the cytosolic domain. Some activated receptors are monomers with guanine cyclase activity and can generate the second messenger cGMP **(left).** The receptors for many grown factors have intrinsic protin-tyrosine kinase activity **(right)**. Ligand binding to most such receptor tyrosine kinase (RTKs) causes formation of an activated homodimer, which phosphorylates several residues in its own cytosolic domain as well as certain substrate proteins.

2.4 MAJOR TYPES OF SIGNAL TRANSDUCTION CASCADES/PATHWAYS

(i) **The Ras pathway:** Ras family proteins are small membrane-associated proteins whose activity depends upon a bound guanosine nucleotide cofactor.

(ii) **The MAP kinase signaling cascade:** MAP kinase (mitogen-activated protein kinase) extracellular-signal regulated kinase (a serine/threonine protein kinase) activated by many growth factors;

(iii) **Stress activated kinases:** Stress-activated protein kinases (SAP kinases) are serine/ threonine kinases, related to MAP kinase which activate a variety of target proteins within the cell.

2.5 SECOND MESSENGERS

Some signal transduction pathways don't involve the direct transfer of external signal to the genome, but utilized more indirect means of influencing gene expression, provided by second messengers.

Many of the signals arriving at the cell surface, for instance, cause the cell to either divide or withdraw from the cell cycle. Others induce expression of characteristic groups of genes which protect the cell from stress. For this reason, early signaling pathways converge into a small number of intracellular signalling networks, and this allows the cell to convert the complex information arriving at the cell surface into simple biochemical signals in the cytoplasm. The molecules involved in this process are termed **second messengers** e.g. Ca^{2+}, cAMP.

The second messenger system is analogous in a way in which glucose modulates the catabolite activator protein by influencing cAMP levels in bacteria cells (see gene regulation in prokaryotes). The cellular levels of cAMP control the activities of various other enzymes, e.g. protein kinase A, is stimulated by cAMP. The actively of protein kinase A is to phosphorylate and hence activate a transcription factor, CREB. This is one of several proteins that influence activity of a variety of genes.

The calcium signaling pathway (Fig. 2.6) involves calcium-binding proteins which when activated by calcium, associate with inactive proteins and stimulate them, thus effecting a number of downstream pathways in gene expression. The annexins are calcium – dependent membrane-binding proteins which may recognize cytoskeletal elements of the cell. The major regulatory calcium-binding proteins include calmodulin (CaM) and troponin C.

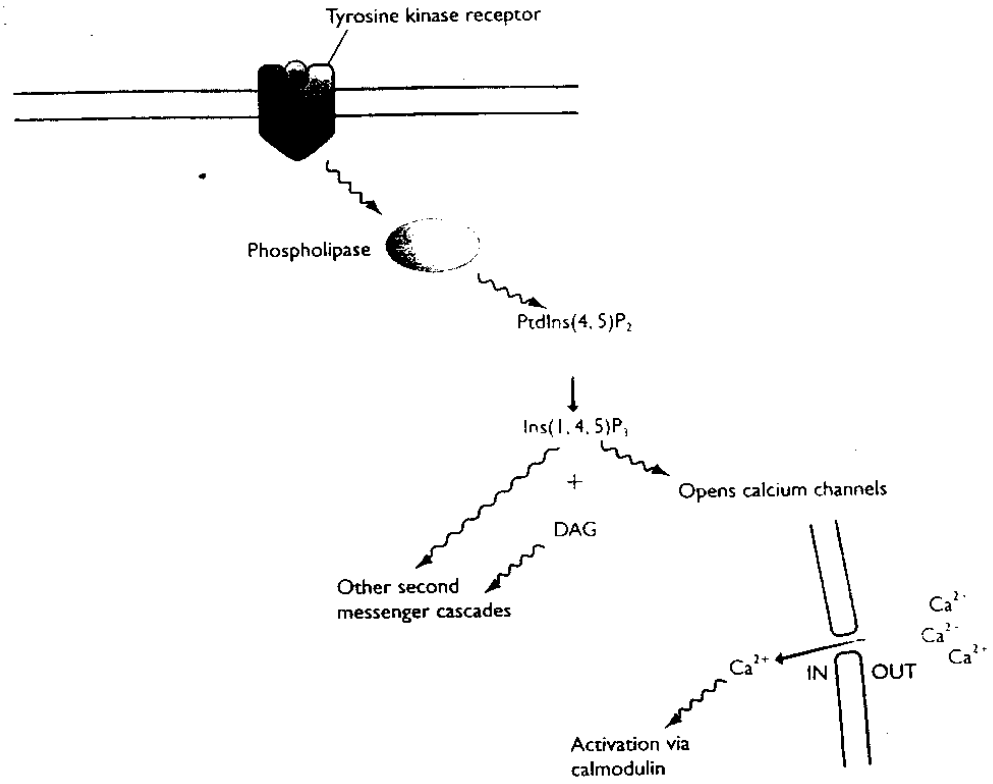

Fig. 2.6.Induction of the calcium second messenger system

2.6 SIGNAL DELIVERY

Signal delivery can be defined as the end of the signal transduction pathway, where the components of the cell which are targets for the initial signal are phosphorylated. Phosphate groups are a universal signaling currency, causing changes to protein shape and therefore activity. Signals mediate their ultimate effects in two ways:

1) by modulating the activity of a protein already present in the cell.

2) By modulating the activity of a transcriptional regulator or translational regulator and thus influencing gene expression.

Many signal transduction pathways terminate at the nucleus. The downstream targets may be components of the cell cycle machinery, allowing control of cell growth or transcription factors, which elicit specific patterns of gene expression through response elements on target genes. Many transcription factors have been shown to be activated by specific signaling pathways and in many cases, the diversity of response is increased by the availability of different isoforms of transcription factor components, e.g. copper regulated gene expression (Fig. 2.7).

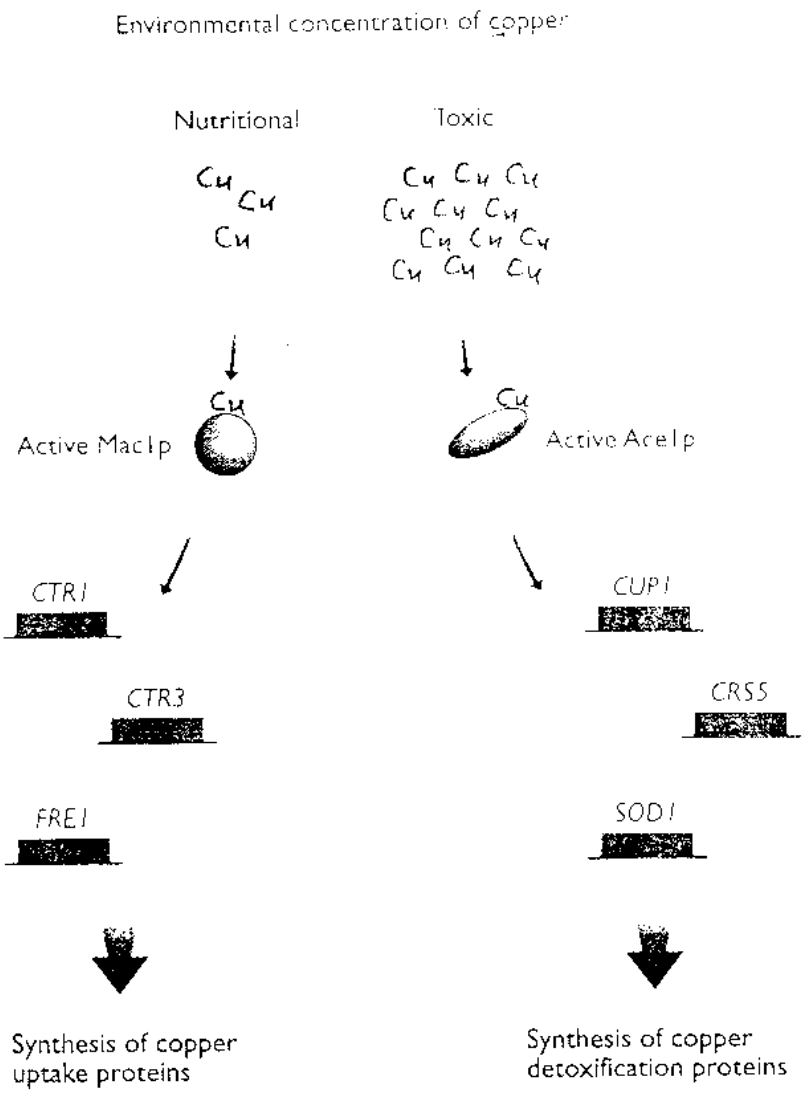

Fig. 2.7 Copper-regulated gene expression in *Saccharomyces cerevisiae*

2.7 SUMMARY

Cells respond to the environment by regulating the activity of proteins and thus alter the patterns of gene expression. Different types of signaling are involved. The multitude of cell surface receptors involves in signalling fate under four classes. The cell-surface receptors with the examples are described. The major pathways of signal transduction and the significance of second messengers in cell signalling/gene regulation are also mentioned in this lesson.

2.8 MODEL QUESTIONS

1. What is signal transduction? How is it carried out at the cell level?
2. Describe the various signal transduction mechanisms operating at the cellular level?
3. Write critical/short notes on:
   a) ion-channels
   b) signal transduction pathways
   c) second messengers

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M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-II

Lesson 3

The Genetics of Cancer

3.0 **OBJECTIVE:**

To introduce an about the genes causing cancer viz., oncogenes and protooncogenes. The viral oncogenes, role of tumor suppressor genes, genetic defects in the mechanism of cell cycle, chromosomal translocations leading to cancer are described here.

3.1 INTRODUCTION

Cancer is a genetic disorder involving dynamic change in the genome, leading to uncontrolled cell growth. This disease involves genetic alterations varying from point mutations to obvious changes in the chromosomal complement. Cancer arise when critical genes are mutated. The malfunctions are triggered by environmental factors such as diet, excessive exposure to sunlight or chemical pollutants. When the cells detach from a tumor and invade surrounding tissues, the tumor is malignant, tumors spread in the body, forming secondary tumors. This process is called *metastatis*. When the cells do not invade surrounding tissue, the tumor is benign.

The morphology and growth properties of tumor cells differ from those of the normal cells. The mutations cause these differences, which was proven by transformation experiments with a line of cultured mouse fibroblast cells. These cells normally grow only when attached to the plastic surface of a cultural dish. They grow only as a monolayer and on contact with other cells become arrested in G_O. When DNA from a human bladder carcinoma or mouse sarcoma is added to cultured 3T3 cells, about 1 in a million of cells incorporated a particular segment of the carcinoma DNA. The progeny of this affected cell can form a cluster of a cells with different shapes. Such transformed cells show properties similar to those of malignant cells (Fig. 3.1).

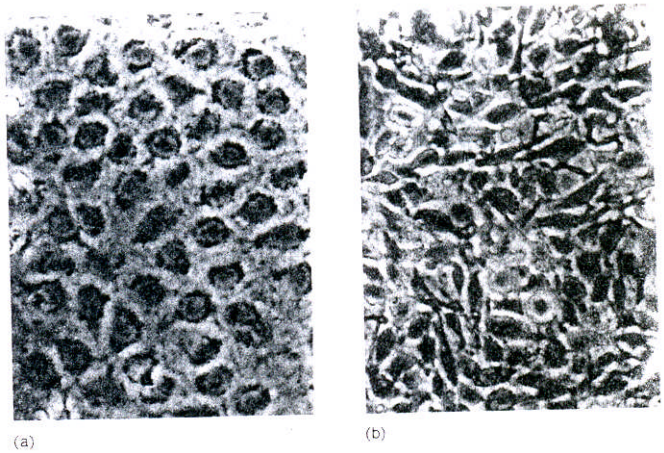


Fig. 3.1 Confluent layers of (a) normal mouse 3T3 cells and (b) 3T3 cells transformed by polyoma virus. The transformed cells not only have a different shape, but also grow in less-organized fashion than normal cells.

The development of malignant tumor (tumorigenesis) is a multistep process characterised by a progression of genetic alterations in a single line of cells that makes the cells increasingly less responsive to the body's normal regulatory machinery and are able to invade normal tissues. Even after they have become malignant, cancer cells continue to accumulate mutations that confer new properties on the cells, making them even more dangerous.

3.2 TYPES OF CANCER

Cancers occur in many types of cells – of nearly 300 types of cells in the human body, about 200 different types of human cancers are known.

Depending on type of cells involved in cancer development, they are named as follows: The major types are e.g.

Leukemias – Bone marrow cells
Lymphomas – Lymphnodes and spleen.
Sarcomas – mesoderm of muscle, bone cartilage.
Carcinomas – epithelial tissue such as glands, breast, genital and respiratory systems etc.

3.3 CANCER AND CELL CYCLE

In a healthy individual the entry of cells into cell cycle and further progression are precisely controlled events / check points (Fig. 3.2) (See cell cycle – Ist year lesson). Cancer is a group of diseases in which cell growth and division are unregulated. This lack of regulation is due to mutations in genes whose protein products are involved in the control of the cell cycle. The cyclins and cyclin dependent kinases (CDKs) etc., are the proteins of the control system (Fig. 3.2). Those proteins check cell-cycle progression, and hold cells in quiscence or even lead cells to commit suicide and thus prevent cells from becoming cancerous. Alteration of any one of these may lead to the loss of control over cell-cycle and the cells may become cancerous. In tumor cells, the check points are deregulated since they lower the availability of cyclin / CDK complexes and ultimately resulting cancer.

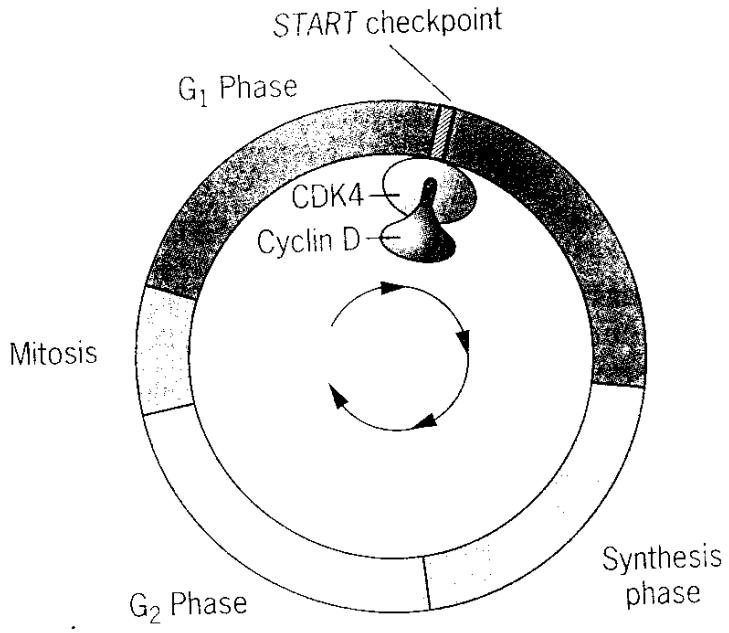


Fig. 3.2 A schematic view of the START checkpoint in the mammalian cell cycle. Passage through the checkpoint depends on the activity of the cyclin D/CDK4 protein complex

3.3.1 Genetic basis of Cancer Development

Cancer researchers have identified two broad classes of genes causing cancer. *I. oncogenes* – a diverse group of genes whose products play important roles in the regulation of biochemical activities within cells and cause cancer.

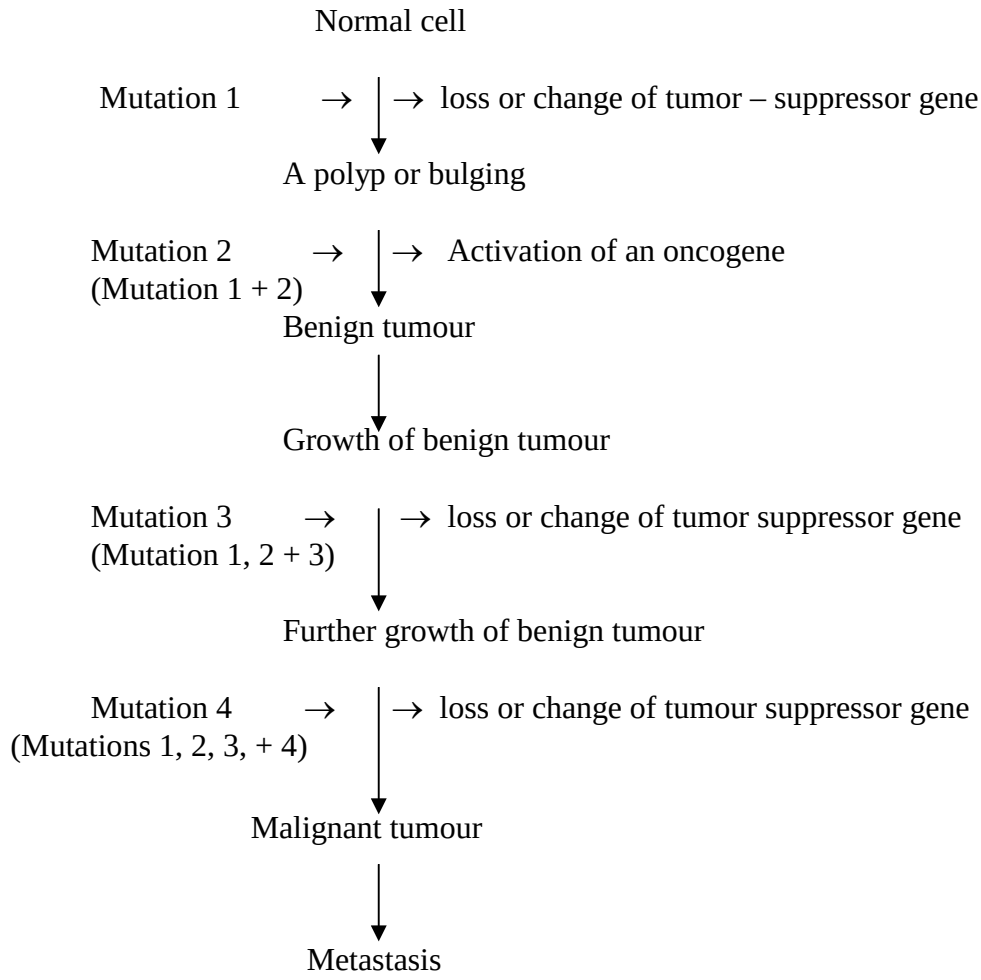
3.3.2 Tumor suppressor genes

They are also called anti-cancer or anti-oncogenes. They act by suppressing malignant growth. But in homozygous recessive state, cancer develops.

General Scheme of Oncogenesis

A mutation in one cell would give it a slight growth advantage. One of the progeny cells would thus undergo a second mutation that would allow its descendants to grow more uncontrollably and form a small benign tumor. A third mutation in a cell within this tumor would allow it to outgrow others. An additional mutation in one of these cells would allow its progeny to escape into blood and start secondary cells. Since decades are required for this multiple mutations to occur, the exponential increase in cancer incidence with age is predicted (Fig. 3.3).

Fig. 3.3 General scheme of oncogenesis – Multi-hit model



3.4 ONCOGENES

Fundamental insight into genetic basis of cancer come from the study of tumor inducing viruses (RNA viruses). The first tumor inducing virus was discovered by Peyton Rous 1910 in

chicken. Of the four genes this virus contain (**gag, pol, env** and **grc**). Src gene is able to induce tumors. The genes of virus such as v-src that cause cancer are called **oncogenes**.

Thus oncogenes (Table 1) may be of viral origin (introduced into human cells when such tumor-inducing retroviruses infect) or may be constituents of normal genome which have undergone mutations.

Table 1 Oncogenes, Their Origin and Their Protein Products

Oncogene	**Virus and Species of Origin**	**Function of Products**
abl	Abelson murine leukemia virus (mouse)	Tyrosine kinase
*fes**	ST feline sarcoma virus (cat)	
*fps**	Fujinami sarcoma virus (chicken)	
fgr	Gardner-Rasheed feline sarcoma virus (cat)	
ros	UR II avian sarcoma virus (chicken)	
src	Rous sarcoma virus (chicken)	Tyrosine kinase
yes	Y 73 sarcoma virus (chicken)	
erbB	Avian erythroblastosis virus (chicken)	Tyrosine kinase
fms	McDonough feline sarcoma virus (cat)	Growth factor
*raf**	3611 Murine sarcoma virus (mouse)	
*mil(mht)**	MH2 virus (chicken)	
mos	Aivan myeloblastrosis virus (chicken)	Protein kinase
sis	Siian sarcoma virus (woolly monkey)	Growth factor
Ha-ras	Harvey murine sarcoma virus (rat)	Bind guanosine triphosphate
fos	FBJ osteosarcoma virus (mouse)	Bind DNA, controls transcription
myb	Avian myeloblastosis virus (chicken)	DNA binding, transcription regulation
myc	MC29 myelocytomatosis virus (chicken)	
erbA	Avian erythroblastosis virus (chicken)	DNA binding
ets	E26 virus (chicken)	
rel	Reticuloendotheliosis virus (turkey)	Bind, DNA – transcription factor
ski	Avian SKV770 virus (chicken)	
jun	Avian sarcoma virus 17 (chicken)	Transcription factor

**fex* and *fps* are feline and avian versions of the same oncogene; *raf* and millmht are murine and avian oncogene counterparts.

3.4.1 Viral oncogenes

Some of the RNA containing viruses/retroviruses introduce their genes into host chromosomes when they infect the cells (Fig. 3.4). Some of the genes (V-sis for example from similar sarcomes virus)encode growth factors. These transforms normal cells into cancerous state. Each type of viral oncogene seems to encode a protein that plays a key role in regulating the

expression of several cellular genes. Thus converting normal cellular oncogenes (**protooncogenes**) to oncogenes. The cellular homologue of v-Src is therefore c-Src.

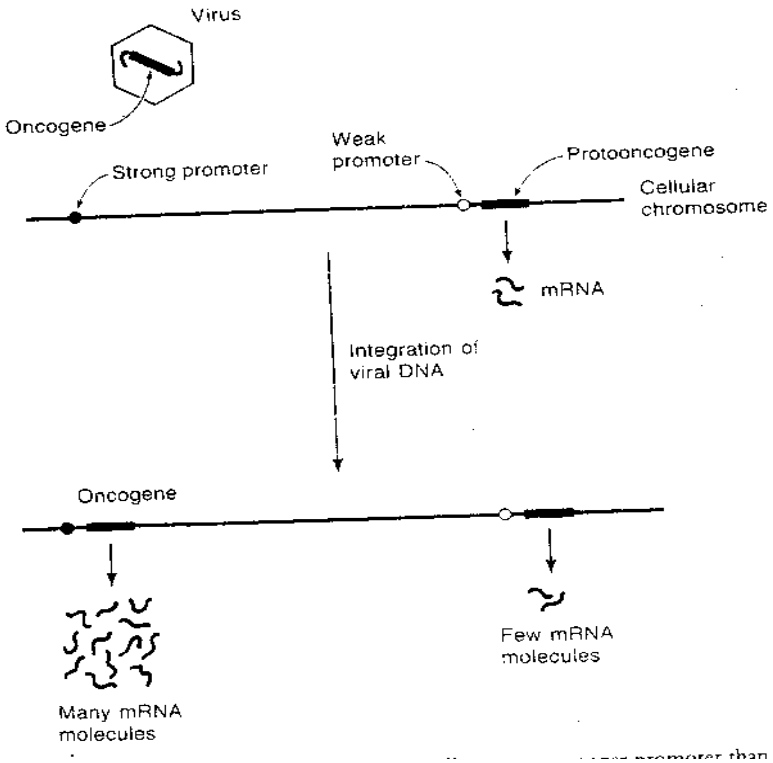


Fig. 3.4 One way by which a viral oncogenes whose coding sequence is the same as that of its cellular counterpart could produce excess product. The viral gene is adjacent to a stronger promoter than the cellular gene, an alternative is that the strong promoter could be in the viral DNA.

3.4.2 Proto-oncogenes

Cells possess a variety of genes such as **proto-oncogenes**, that have the potential to subvert the cell's own activities and push the cell toward the malignant state. Proto-oncogenes encode proteins that have various functions in a cell's normal activities. Proto-ongogenes can be converted into oncogenes are thus cellular oncogenes. Proto-oncogenes can be converted into oncogenes (i.e., activated) by any of the following mechanisms.

1. The gene can be mutated in a way that alters the properties of the gene product so that it can no longer carry out its normal activity (Path A).

2. A mutation in a nearly regulatory sequence can alter the expression of the gene, so that an excessive quantity of the gene product is produced (Path b).

3. A chromosome rearrangement can occur that brings a DNA sequence from a distant site in the genome into close proximity of the gene, which can either alter the expression of the gene or the nature of the gene product (Path c).

Any of these genetic alterations can cause a cell to become less responsive to normal growth controls, causing it to behave as a malignant cell. Oncogenes act dominantly, that a single copy of an oncogene can cause the cell to express the altered phenotype, regardless of whether or not there is a normal, inactivated copy of the gene on the homologous chromosome. There are two types of genes responsible for tumor formation. As long as a cell has its full complement of tumor-suppressor genes, it is thought to be protected against the effects of an oncogene. Most tumors contain alterations in both tumor suppressor genes and oncogenes, suggesting that the loss of a tumor suppressor function within a cell must be accompanied by the conversion of a proto-oncogene into an oncogene before the cell becomes fully malignant. Even then, the cell may not exhibit all of the properties required to invade surrounding tissues or to form secondary colonies by metastasis. In general, the number of altered genes increases along with a progression in the malignant virulence of the tumor, e.g. Studies of colorectal carcinomas indicate, for example, that mutations in as many as seven different genes may be necessary for the development of a fully malignant tumor.

3.5 TUMOR SUPPRESSOR GENES

Tumor suppressor genes are anti-cancer or anti-oncogenes. These genes seem to act by suppressing maligrant growth, when they are mutated cells become caner.

In one way or another, most of the proteins encoded by tumor-suppressor genes act as negative regulators of cell proliferation (suppress oncogenes) that is why their elimination promotes uncontrolled cell growth. It was observed among 20 different cancer syndromes, the underlying defect is in a tumor suppressor gene rather than in an oncogene. Tumor suppressor genes were discovered through their association with rare inherited cancers such as **retinoblastoma**.

3.5.1 Retinoblastoma: The development of retinoblastoma (formation of retinal tumors) is presented here as a typical example of oncogenesis. This was explained in 1971 by Alfred Knudson of the University of Texas. **Retinoblastoma** a tumor of retinoblast cells (Fig. 3.5) is inherited as a

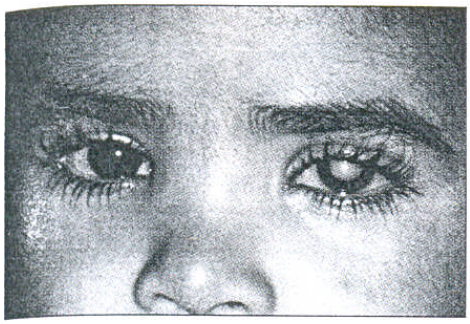


Fig. 3.5 The retinoblastoma tumor suppressor gene. A child with a retinoblastoma tumor in the left eye.

dominant genetic trait because members of high-risk families that develop the disease inherit one normal allele and one abnormal allele. Children who inherit a chromosome, missing the retinoblastoma gene inherit a strong disposition towards developing retinoblastoma rather than the disorder itself. Knudson proposed that the development of retinoblastoma – requires that both copies of the RB gene of a retinal cell be either eliminated or mutated before the cell can give rise to a retinoblastoma. In other words, the cancer arises as a result of two independent "hits" in a single cell. In cases of sporadic retinoblastoma, the tumor develops from a retinal cell in which both copies of the RB gene have undergone successive spontaneous mutation. The chance that both alleles of the same gene will be the target of inactivating mutations in the same cell is extremely less. In contrast, the cells of a person who inherits a chromosome with an RB deletion are already halfway along the path to become malignant. A mutation in the remaining RB allele in any of the cells of the retina, produces a cell that lacks a normal RB gene and thus cannot produce a functional RB gene product. This explains why individuals who inherit an abnormal RB gene are so highly predisposed to develop the cancer.

The second "hit" fails to occur in approximately 10 per cent of these individuals who do not develop the disease. Knudson's hypothesis was subsequently confirmed by examining cells from parents with an inherited disposition to retinoblastoma and finding that, as predicted, both alleles of the gene were mutated in the cancer cells. Individuals with sporadic retinoblastomas had normal cells that lacked RB mutations and tumor cells in which both alleles of the gene were mutated (Fig. 3.6). People who suffer from the inherited form of retinoblastoma are also at high risks of developing other types of tumors later in life, particularly soft tissue sarcomas. Recent research has revealed that RB tumor suppressor protein plays a key role in regulation of cell cycle.

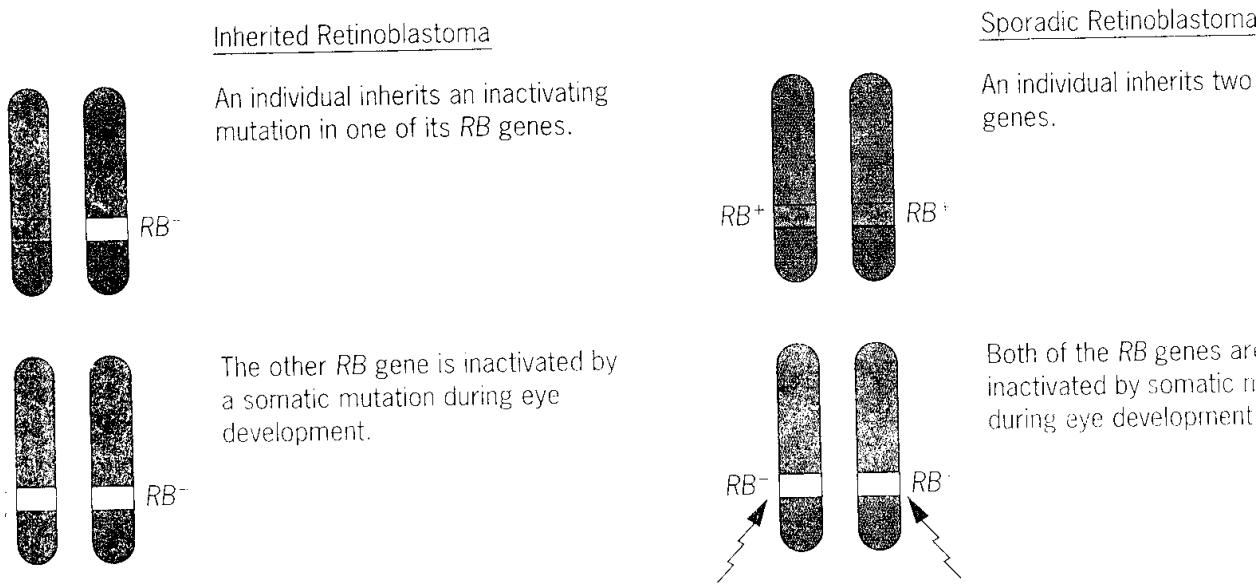


Fig. 3.6 Knudson's two-hit hypothesis to explain the occurrence of inherited and sporadic cases of retinoblastoma. Two inactivating mutations are required to eliminate the function of the *RB* gene.

3.5.2 The role of pRb and P53 in regulating the cell cycle

pRb: The protein encoded by the RB gene (pRb), helps to regulate the passage of cells from the G_1 stage of the cell cycle into S phase, during which DNA synthesis occurs. The transition from G_1 to S phase is the time of commitment for the cells - once a cell enters S phase, it invariably proceeds through the remainder of the cell cycle and into mitosis. The transition from G_1 to S is accompanied by the activation of many different genes that encode proteins ranging from DNA polymerases to cyclins and histones. Among the transcription factors involved in activating genes required for S-phase activities, are members of the E_2F (proteins coded by genes E_2&F) which are key targets of pRb. A cell that losses pRb activity as the result of RB mutation would be expected to lose its ability to activate E_2F, thereby cell can't enter into S phase. Thus they regulate the cell cycle.

P53

P53 Protein plays key role in cellular responses to stress. It is a tumor suppressor protein. In normal cells, the level of p53 is low but when cells are treated with radiation, p53 increases to repair the damaged DNA due to radiation.

The P53 gene commonly regarded as the 'Guardian of the genome' may have more to do with the development of human cancer than any other component of the genome. The gene gets its name from the product it encodes, P53, which is a polypeptide having a molecular mass of 53,000 daltons. In 1990, the gene P53 was recognised as the tumor-suppressor gene that when absent, is responsible for a rare inherited disorder called Li-Fraumeni Syndrome in which several types of cancer may develop. The elimination of P53 function is an important step in the progression of many cancer cells toward the fully malignant state. More than a thousand different P53 mutations have been identified among human tumor samples, indicating that the proper functioning of the protein is very sensitive to even slight changes in amino acid sequence.

Cell cycle arrest is not only a way that P53 protects an organism from developing cancer. Alternatively, P53 can direct a genetically damaged cell along a pathway that leads to death by apoptosis, thereby reducing the malignant potential of the body of cells.

During 'S' phase of cell cycle at the time of DNA replication, DNA mismatch repair requires, the cooperative efforts of a number of proteins including proteins that recognize the lesion, to remove a portion of the strand containing the lesion, and replace the missing segment with complementary nucleotides. If any of these proteins are defective, the affected cell can be expected to display an abnormally high mutation rate, which in turn increases its risk of becoming malignant. The first hard evidence that a defect in mismatch repair might be a factor in the development of cancer came in 1993 in studies on the cells of patients with the most common hereditary form of colon cancer, called hereditary non-polyposis colon cancer (HNPCC).

3.6 CHROMOSOMAL REARRANGEMENTS AND CANCER

Chromosomal abnormalities, both structural (mostly interchanges) and numeral (mostly aneuploidy) are common in tumor cells. These abnormalities are not common to all tumors but each tumor has its own set of anomalies. The first discovered chromosomal abnormality in human tumors is the 'Philadelphia chromosome' which is found in hematopoietic cells of nearly all patients with the disease **chronic myelogenous leukemia**. This chromosome results from a translocation between chromosomes 9 and 22. As a result, the fusion polypeptide cause uncontrolled division of white blood cells to become cancerous (Fig. 3.7).

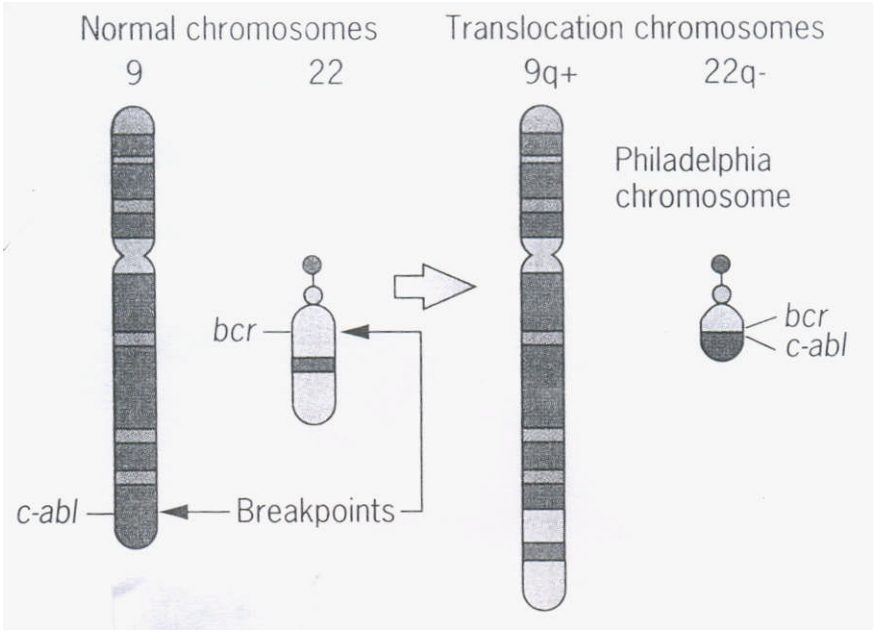


Fig. 3.7 The reciprocal translocation involved in the Philadelphia chromosome associated with chronic myelogenous leukemia.

Another reciprocal translocation is seen in the cancer type called Burkitt's lymphoma. The translocation involves chromosomes 8 and 14 (Fig. 3.8). Due to this translocation, a gene called c-myc originally located on chromosome 8 is moved to a place on chromosomes 14 and near to an antibody gene enhancer. Consequently, rearrangements results in the over expression of C-myc oncogene that produce immunoglobulin heavy chain in β-lymphocytes and such over expression causes those cells to become cancerous.

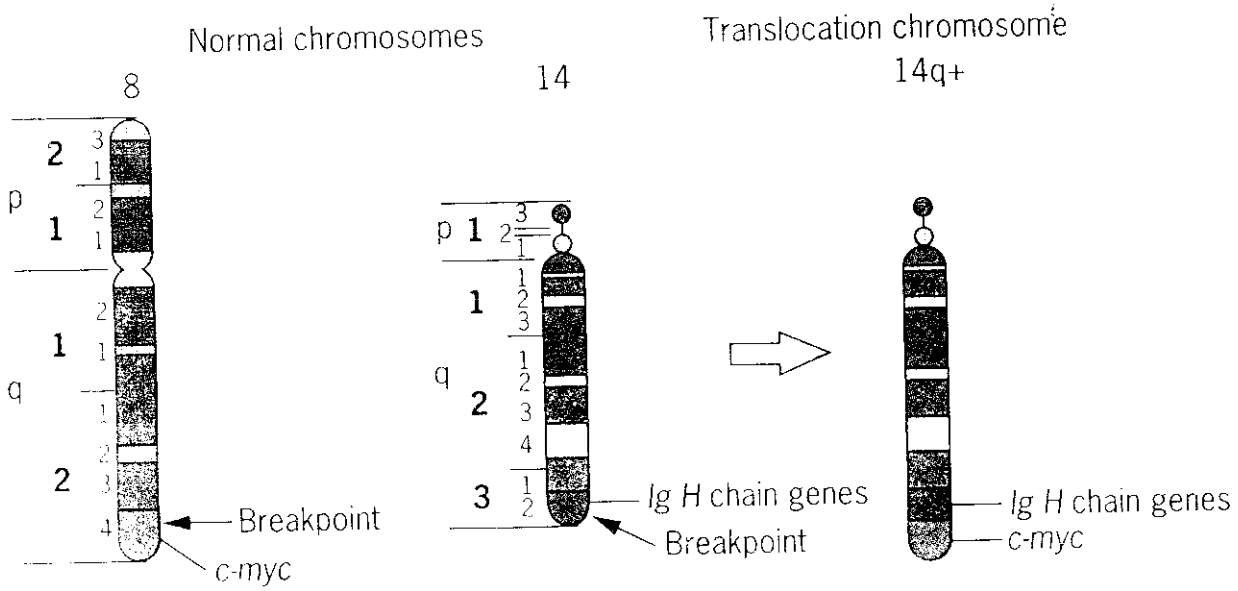


Fig. 3.8 A recipient Translocation chromosome Burkitt's lymphoma.

Many therapeutic protocols have shown encouraging results in a number of different malignancies. However, no strategy has been universally successful as yet e.g. **Radiation therapy** – By radiation treatment the tumor cells are killed. **Chemotherapy** – The use of certain chemicals, adriamycin, arabinosylcytosine have been effective in treatment of 70 per cent of cancers. **Immuno therapy** – It restores body's defensive mechanism. **Gene therapy** – Through recombinant DNA technology (somatic-cell gene therapy) modified cultured cells are replaced in the patient to rectify the defects due to cancer.

3.7 SUMMARY

Cancer result from genetic malfunctions. Genetic basis of cancer have come from the study of tumor-inducing viruses. Genetic inactivation or loss of tumor suppressor genes and activation of proto-oncogenes to oncogenes which are two main changes underlying cancer development are outlined. These genetic changes are associated with production of specific proteins. If proteins of DNA mismatch repairs are defective there is risk of malignancy. All these effect ultimately the cell cycle control points, leading to uncontrolled number of cell divisions resulting in the formation of cancerous cells. The role of pRB and p53 proteins as tumor suppressors are mentioned in this lesson. The types of human cancer associated with chromosome rearrangements are described here.

3.8 MODEL QUESTIONS

1. Explain the genetic basis of cancer.
2. Distinguish between protooncogenes, oncogenes and tumor suppressor genes. What is their role in the development of cancer.
3. Write short notes on:
   i) Philadelphia chromosomes
   ii) P53
   iii) Retroviruses

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M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-II

Lesson 4

TRANSPOSABLE ELEMENTS – AN OVERVIEW

4.1 INTRODUCTION

A typical gene has a specific fixed position in the genome. Contrary to this general feature of a gene, evidences were shown where movement of genetic elements from one place to other across the genome was documented in 1948 by McClintock in maize. The present topic deals with such mobile DNA segments/jumping genes called transposable elements.

The mobile genetic elements were discovered in maize by renowned lady cytogenetist, Barbara McClinktock in 1948 without the aid of tools of molecular genetics. She was awarded Nobel Prize in 1983 for her work (Fig. 4.1). The elements discovered by her are Activator (Ac) and Dissociator (Ds) for which she was award Nobel Prize in 1983. Through genetic analysis, McClintock showed that the activities of these elements are responsible for the striping and spotting of colour on maize kernals (Fig. 4.2). Mutations caused by insertion of a transposable elements in a given gene are somatically ustable, due to precise excision of the transposable element. Many years later, these elements were isolated and their molecular structure was determined. Extensive research since the time of McClintock has indicated that these mobile genetic elements (originally named as controlling elements by McClinktock) are present in the genomes of many organisms –

both prokaryotes and eukaryotes and were identified as transposons. The situation changed in 1960s and 1970s when transposition was discovered in bacteria and *Drosophila*. At this juncture, the scientific world awake to the broad significance of McClintock's ideas.

Fig. 4.1 Barbara McClintock

Fig. 4.2 Color variation among kernels of maize. Studies of the genetic basis of this variation led to the discovery of transposable elements.

4.2 TRANSPOSONS

They influence gene expression and differ in structure, mechanism of transposition, distribution etc.

The movement of these elements has effects on the phenotype of the organism. Hence, they are also termed as selfish DNA. When we map a gene today, we expect to see it in same place tomorrow. However, the discovery of these transposable genetic elements has modified this view to some extent.

4.3 CLASSIFICATION

Transposable elements fall into two major classes based on their mechanism of transfer.

i) Class I transposable elements (Retrotransposous)

The reversal in the flow of genetic information has led geneticists to call these elements **retrotransposons**. They are retrovirus like elements and retroposons. The first class resemble chromosomes of group of viruses that depend on reverse transcription for their propagation, the retroposons move through an RNA molecule that is reverse transcribed into DNA. They do not have inverted or direct repeats as integral parts of their termini but show their origin as reverse transcripts of polyadenylated RNAs.

ii) Class II transposable elements (transposons)

They transpose DNA directly either by excision and integration or by a replicating process whereby a copy of the element is left at the original site and another is integrated at a new site.

Transposons are mobile genetic elements. They are segments of DNA that are not autonomous and can insert at random into the genome, plasmids or bacterial chromosomes independent of host cell recombination system.

Transposable elements are active or autonomous if they encode the functions required for their own transposition. For transposition, the minimum requirements are the enzyme transposase and the cis-acting sites it recognizes, allowing the element to determine the boundary between itself and host DNA.

For retro elements, reverse transcriptase is required in addition to transposase (which is termed ‘integrase’ in this case).

Other transposable elements, described as defective, lack enzyme functions but retain the cis-acting sites and may be mobilized in trans by an active element. In other words, elements

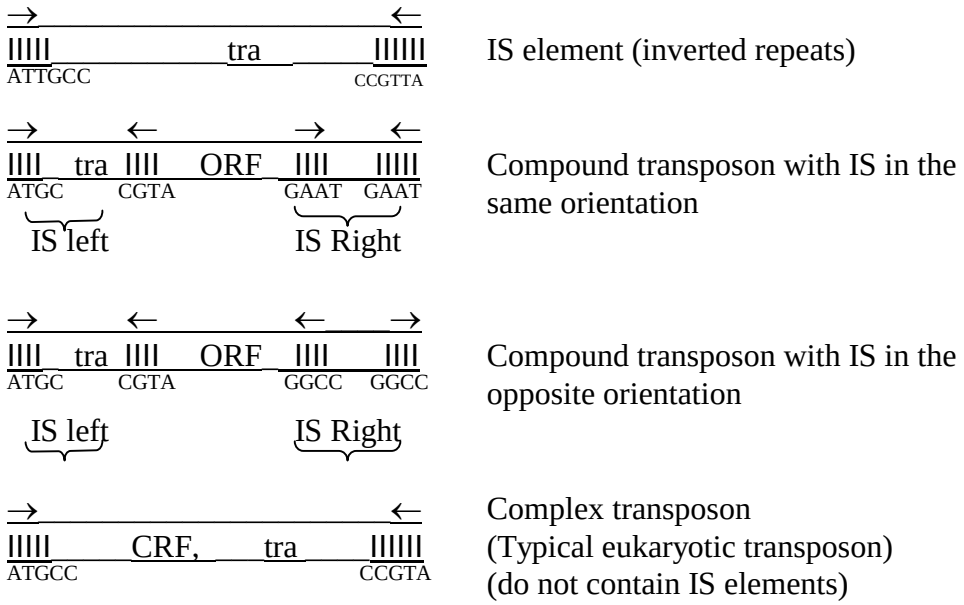
mobilized in trans are described as non-autonomous or passive or defective. The active and defective elements form families of related sequence.

4.3.1 Structure

All transposons have a conserved structure comprising one or more ORFS (opending reading frame sequence one encoding transposase) flanked by inverted terminal repeats (ITRs) (Fig. 4.3). The terminal repeats are necessary for transposition and are the substrates recognized by the transposase.

All eukaryotic transposons resemble bacterial IS (Insertion sequence) elements in that they encode only the functions required for transposition. Most has a canonical structure comprising a central region flanked by short ITRS [Inverted terminal repeat sequence].

Fig. 4.3 Structure of Transposons



4.3.2 Transposable elements in bacteria (IS sequences)

Genetic instabilities in bacteria have led to the identification of transposable elements. They were studied at molecular level during 1967. The transposable elements discovered in bacteria were

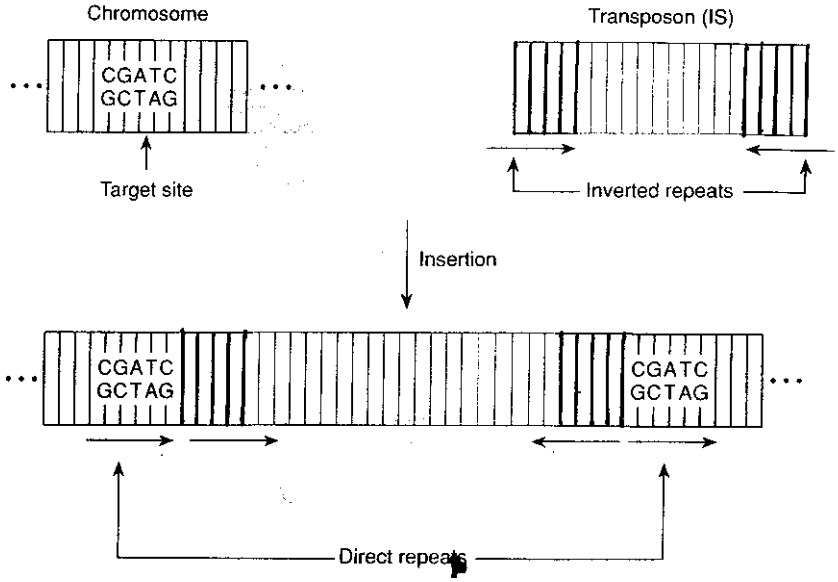


Fig. 4.4 An IS element inserted into a target site in a bacteria chromosome creates a direct repeat on either side of the IS element.

called insertion sequences or IS elements which are autonomous units. They vary in size. Features of some IS elements are given in Table 1.

Table 1. Features of some IS elements

Name	Element size (bp)	TIR left/right (bp)	TSDs (bp)	Target site preference
IS1	768	20/23	9	AT rich terminal G or C
IS2	1327	32/41	5	Hot spots in P_2 genome
IS3	1258	29/40	3	?
IS4	1426	16/18	11-13	$AAAN_{20}TTT$
IS5	1195	16	4	Hot spots
IS10	1329	17/22	9	NGCTNAGCN
IS50R	1531	9	9	Hot spots
IS91	-1800	8/9	0	?

Note: ITRs – Inverted terminal repeats; TSD – Target site duplication; bp – base pairs

i) IS elements (Insertion sequences) – They were discovered by their ability to cause stable but strongly polar mutation in *E. coli*. About 100 are known – all are small (< 2.5 kbp), and have a central unique region flanked by imperfect ITRS. The central region may have 1-3 ORFS one of which codes transposase but there are no other genes. Different IS elements differ with respect to their target preference and the size of the target site duplication they generate.

ii) Composite transposons: They comprise a central region containing non-essential genes (e.g. antibiotic resistance) flanked by two IS elements (Fig. 4.5). The IS elements may lie in the same or opposite directions so that the total structure appears to have either long direct or long inverted repeats. The flanking of IS may be identical or different. The transposase function (the substrates for transposition) is provided by one or both IS elements which cooperate to transpose the intervening DNA. Besides, each of these, IS elements can mobilize individually. The maintenance of the transposon (Transposon coherence) reflects selective pressure for the phenotype conferred by the intervening DNA.

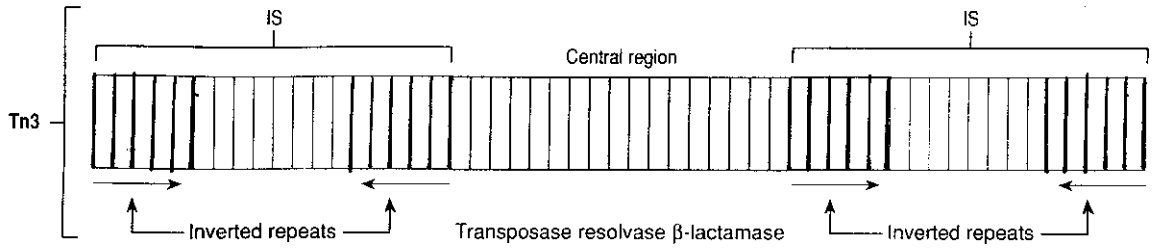


Fig. 4.5 A composite transposon consists of a central region flanked by two IS elements. Transposon Tn3 contains the transposase and resolvase enzyme genes a well as the bacterial gene. β-lactamase, which protects the cell from the antibiotic ampicillin.

iii) Complex transposons – They don't contain IS elements. They have inverted terminal repeats, long with 38-40 nucleotide base pairs. They are larger than IS elements and usually contain accessory genes as well as genes needed for transposition e.g. Tn3.

Transposon families in eukaryotes: In eukaryotes transponsons are termed Class II type elements. Most eukaryotic transposons are similar to bacterial IS elements, comprising a central region, coding for transposase, flanked by short inverted repeats. A few examples are described below:

Ac-Ds family: Activator and Dissociation elements of maize represent simple transposable system. Autonomous activator element, mediates the phenomenon of transposition, Ds is a dissociation element induces chromosome breakage, but only when Ac element is present in maize genome. Hence, Ac/Ds system provides explanation for the genetic instability, McClictock has observed with regard to chromosome.

Yeast Ty Elements – Ty is for transponson in yeast. They were discovered by Cameron *et al*., 1979. They were found at different sites in different strains of yeast. The yeast carries 35 copies of Ty elements. The transposition of Ty elements involves an RNA intermediate and hence Ty elements are called retrotransposons.

Drosophila elements – Of all transposable elements of **Drosophila**, the most useful ones to Geneticists are P elements. They are present in 30-35 copies/genome. They are responsible for hybrid dysgenesis (hybrid sterility) when a sperm carrying **p** element fertilizes a non- **p** strain, the **p** element transpose through out the genome disrupting vital genes.

In both Ac-Ds and P-elements, the transposase gene appears to be the only ORF and is interrupted by several introns. In P-elements splicing is differently done in somatic and germ lines, so that transposition occurs only in the germ cells. In Ac-Ds, splicing is constitutive and not restricted to germ line.

4.4 MECHANISM OF TRANSPOSITION

During transposition in majority of cases the process got through a co-integrate state, where there is fusion of the element (Fig. 4.6). Two types of mechanisms are mentioned here.

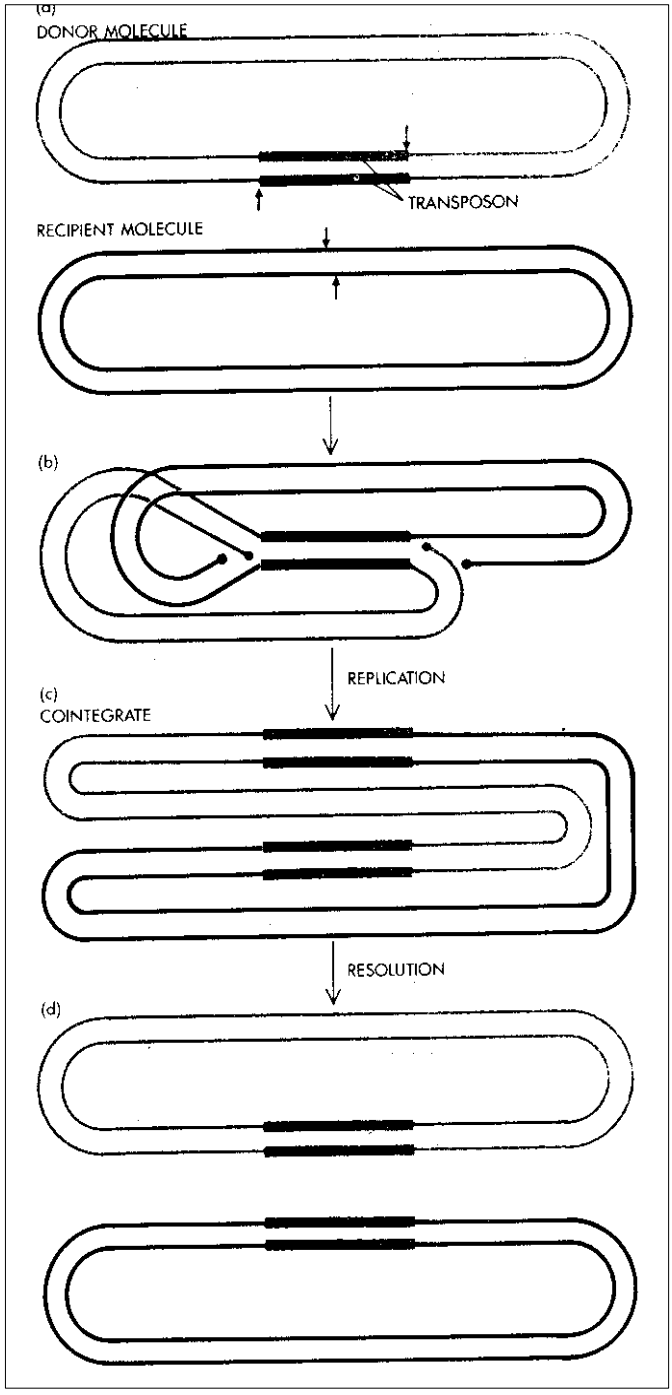


Fig. 4.6 A model for transposition.
(a) Single stranded cuts are made at the ends of the transposon on he donor molecule and staggered cuts 5 to 7 bases apart in the target DNA (recipient molecule). (b) Each transposon end is attached to a protruding target DNA end. Replication from the two forks copies the transposon to produce structure. (c) The resulting "cointegrate" consists of two copies of the transposon in a direct-repeat orientation. (d) A site-specific crossover at the internal resolution or "res" site produces the starting donor molecule and the recipient molecule, now containing a copy of the transposon. →

i) **Conservative/Non-replicative/Simple/Cut and Paste mechanism:** It involves the excision of the element from one site and its integrations at another. It does not increase the copy number of the element (Fig. 4.7A).

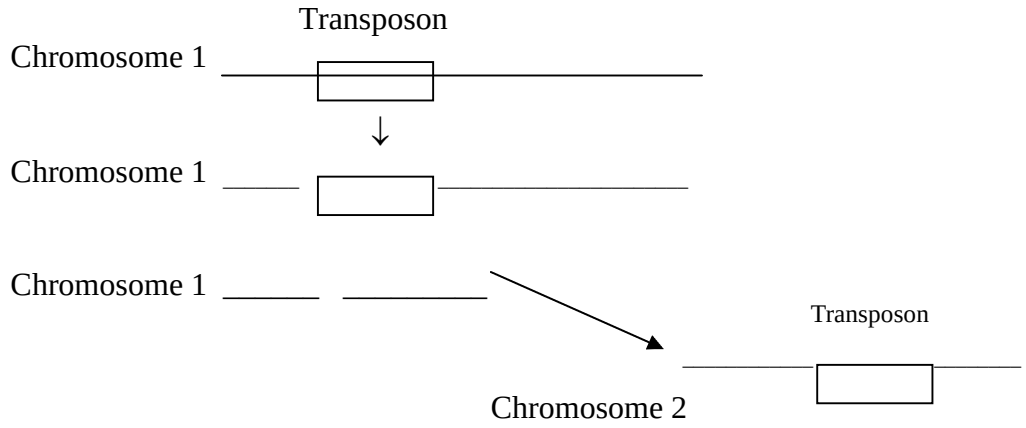


Fig. 4.7A Conservative type of transposition

i) Replicative transposition/non-conservative/Complex type – It involves duplication of the element, one copy remaining at the donor site and one copy integrating at the target site.

Retro-element transposition is necessarily replicative because the element yielding the transcript which is converted into a cDNA for integration remains in the genome (Fig. 4.7B).

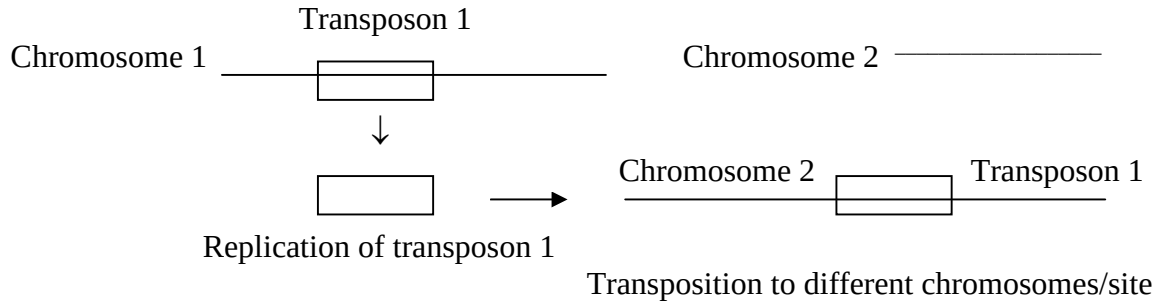


Fig. 4.7B Replicative transposition

4.4.1 Regulation of transposition: Behaving as selfish DNA, successful transposable elements mobilize often and increase their copy number wherever possible. However, unchecked transposition would destroy the host genome and any transposons contained in it. Most transposons, therefore regulate their own transposition. An exception to this rule is the transposing bacteriophage Mu to whom the survival of the host *E. coli* is inconsequential. The virus destroys the host genome by repeated transposition and the multiple copies are then packaged along with host DNA into phage particles.

4.5 APPLICATION OF TRANSPOSONS

(1) **Gene delivery vectors:** (i) Transposon tagging for introduction of genes into bacterial chromosomes by cloning in a transposon and introducing by transformation.

(ii) introducing genes into **Drosophila** by injecting plasmids containing recombinant P-elements into germ cells.

(iii) introduction of genes into plants by deliving recombinant Ac C-Ds elements on Ti plasmid vectors.

(iv) generating transgenic mice using recombinant retroviral vectors.

(2) Induction of Gene Mutations: Transposable elements are responsible for mutations in wide variety of organisms. They affect genes directly by inserting into them leading to major effects on gene expression.

Thus due to their effect on gene expression, transposons contribute to generation of variability in populations and thereby their evolutionary process.

4.6 SUMMARY

Mobile genetic elements are segments of DNA capable of moving from site to site within the genome of the cell. Transposons are the most widely distributed category of these mobile genetic elements. Different types of transposons, families of transposons their structures are given. The process of moving from one site to another is called transposition and is mediated by an enzyme called transposase. This enzyme is coded by the transposon itself. In natural populations, mobile genetic elements contribute to evolution by generating variability. They can be used as vectors for transferring genes and for induction of mutations.

4.7 MODEL QUESTIONS

i) Give an account of the transposable elements.
ii) Write notes on:
   a) Ac-Ds element
   b) IS elements, compound transposons.
   c) Mechanism of Transposition.

1.8 REFERENCE BOOKS

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M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-III

Lesson 1

DNA as genetic material

1.1 INTRODUCTION

Biological systems contain four major types of maromolecules. They are polysacchairdes, fats, proteins and nucleic acids. By 1930, the major question that confronted the biologists in general and genetisists in particular was the chemical nature of genetic material and how it exactly functions. For any substance to function as the genetic material, the following conditions are to be satisfied.

1. The substance should carry the information that determines specific properties (characters) of the organism.
2. It should be able to replicate itself and transfer the genetic information from parent to progeny.
3. It should be stable both physically and chemically so that the information is not altered.
4. It should be capable of undergoing heritable changes without major loss of parental information.

In the early days, it was widely believed that genetic material was proteinacious in nature. This idea was based on the concept that, of all the known intracellular macromolecules, only proteins were sufficiently complex and showed variation in both structure and chemical properties enough to contain (accommodate) the great variety of information required of the genetic substance. Meischer 1868 identified an acidic substance, he isolated from human pus cells called it nuclein. At that time, the importance of the substance was not anticipated.

Three simple experiments but done with great care reversed these earlier notions on the chemical nature of the genetic material and identified beyond doubt, DNA as the genetic material.

1.2 GRIFFITH'S TRANSFORMATIONS EXPERIMENTS

The first experiment was by Frederick Griffith in 1928 on the human pneumonea causing bacteria – *Streptococcus pneumoniae* (*Pneumococcus*). This organism occurs in two major forms.

1.2.1. Smooth (S) type: The cells have a polysaccharide capsule. As a result, its colonies appear smooth. These forms are virulent as they are capable of causing the disease (pneumonea) in the experimental animals.

1.2.2 Rough (R) type: These cells lack a capsule and appear as rough and dull surface colonies. These cells are non-virulent. The distinction between rough and smooth types is genetically controlled and solely dependent on presence or absence of the capsule.

Based on the types of polysauccharides constituting the capsule the smooth type can be further distinguished into S-I, S-II, S-III, S-IV etc. The 'R' and all these types of 'S' are true breeding. Each 'S' type has its specific antigenetic property. Under normal conditions, mutations from smooth to rough are known to occur with frequency of 1 in 10^7 cells and the reverse mutation is much less frequent. It was also established that when these, rough cells (R II) rarely revert to smooth, these revertents are again of the original S-II type.

The results of Griffith's experiments can be summarised as follows (Fig. 1.1):

1. When laboratory mice are injected with live 'R' type bacteria derived from S-II culture, no disease was observed.

2. When the living S-II type were injected, the mice showed disease symptoms.

3. Use of heat killed suspension of 'S' type did not produce any disease.

4. When live R-II bacteria (derived from S-II type by mutation) were injected together with heat killed S-III suspension (dead S-III type bacteria), the mice showed disease symptoms. Following the autopsy of the dead mice, both living R-II and S-III bacterial types could be cultured.

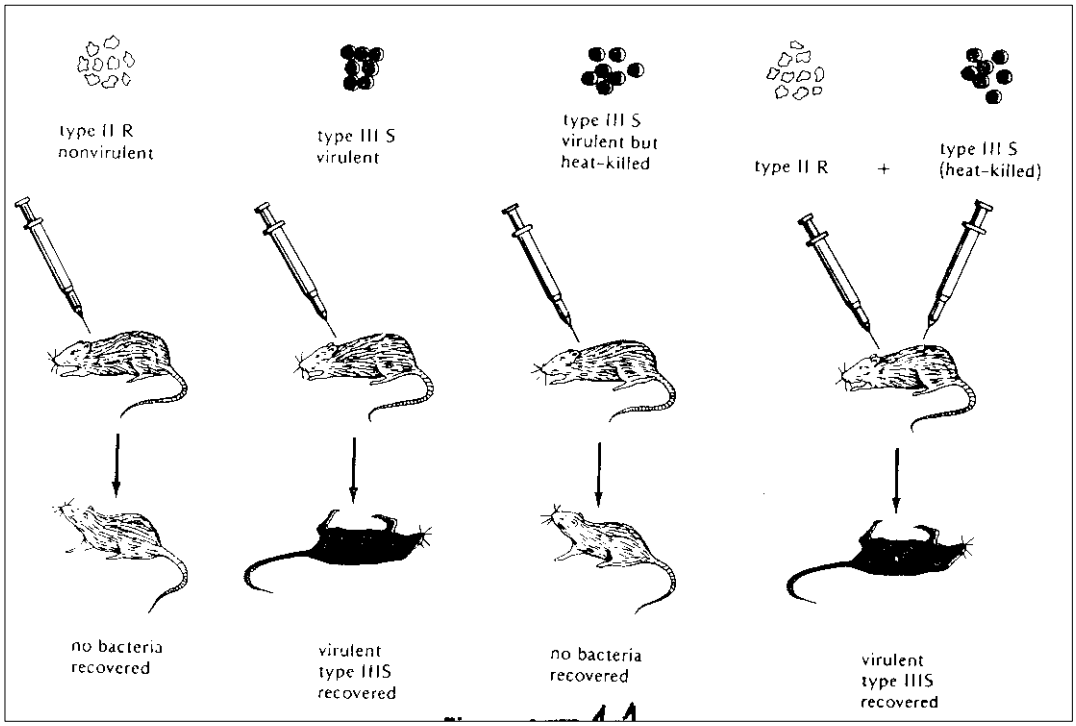

Fig. 1.1 Transformation experiment of Griffith

From these results, it appeared that somehow the dead S-III type were revived as it is observed. The high frequency of such results also excluded the possibility of the occurrence of reverse mutation (r to S) which was of very low frequency. These results were taken to mean at that time as the cases where heat killed cells S-III became revived. It appeared that non-virulent ones or in short, the living R bacterial cells were somehow transformed. Therefore, the Griffith's experiment gradually became known as transformation experiments and turned out to be the first major steps in the identification of the genetic material. Evidence has since been accumulated to show that transformation is of fairly wide occurrence in bacteria and probably occur in the blue green algae as well.

1.3 IDENTIFICATION OF THE TRANSFORMING SUBSTANCE

Sixteen years after Griffith's experiment i.e., in 1944, Avery, Macleod at McCarty repeated successfully the work of Griffith's and further extend it to the level of biochemical analysis of the heat killed suspension of bacteria. When they used cell fractions containing polysacharide capsule or various cell proteins or even Ribonucleic acid, no transformation was observed, only extracts containing DNA were effective. Even highly purified fraction containing DNA + less than 2 parts per 10,000 of protein retained the transforming ability (Fig. 1.2).

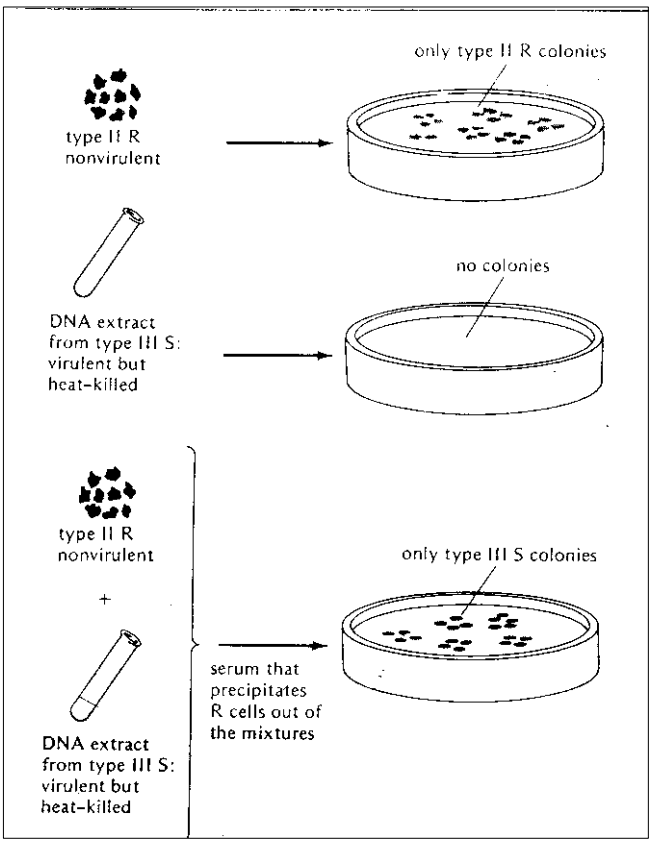

Fig. 1.2 Transformation experiment of Avery, MacLeod, and McCarty

1.4 BLENDER EXPERIMENT BY HERSHEY & CHASE

Additional support for the DNA as the genetic material came from experiments of Hershey and Chase, 1952 using radiactive labelled bacteriophages. There are several different types of bacteriophages that infect the colon bacterium *Escherichia coli*. Electron microscopic studies showed that the phages of *E. coli* have two major parts designated as Head and tail. The head and tail structures are made up of proteins and form the coat of the virus. DNA is found only within the head.

The initial step in phage infection is the attachment or absorption of the tail of the phage to the bacterial cell surface. Phages reproduced within bacteria, the infected bacteria break open or lyse and the phage progeny are released. The protein and the DNA of these new phages are formed from constituents of the bacterial protoplasm, ultimately killing the host bacteria. When a bacteria is infected with a single phage of a given genotype, the progeny that emerged upon lysis of the bacterium are genetically identical to the original infecting phage.

Analysis of the composition of the phages had revealed that they are composed primarily of protein and DNA. Phosphorus is one of the major constituents of DNA but sulphur is not. The protein on the other hand, has very little phosphorous but it does contain sulphur. With this knowledge, Hershey and Chase grew host bacteria in a medium containing either the radioactive isotope of sulphur (S^{35}) or that of phosphorus (P^{35}). During the growth process, the bacteria incorporated these isotopes, thereby labelling their protoplasmic constituents with radioactive sulphur or phosphorus. The labelled bacteria were then infected with phages and after lysis, the progeny phages were released. These progeny were collected and found to be labelled with the specific radioactive isotope of the host bacteria (Fig. 1.3).

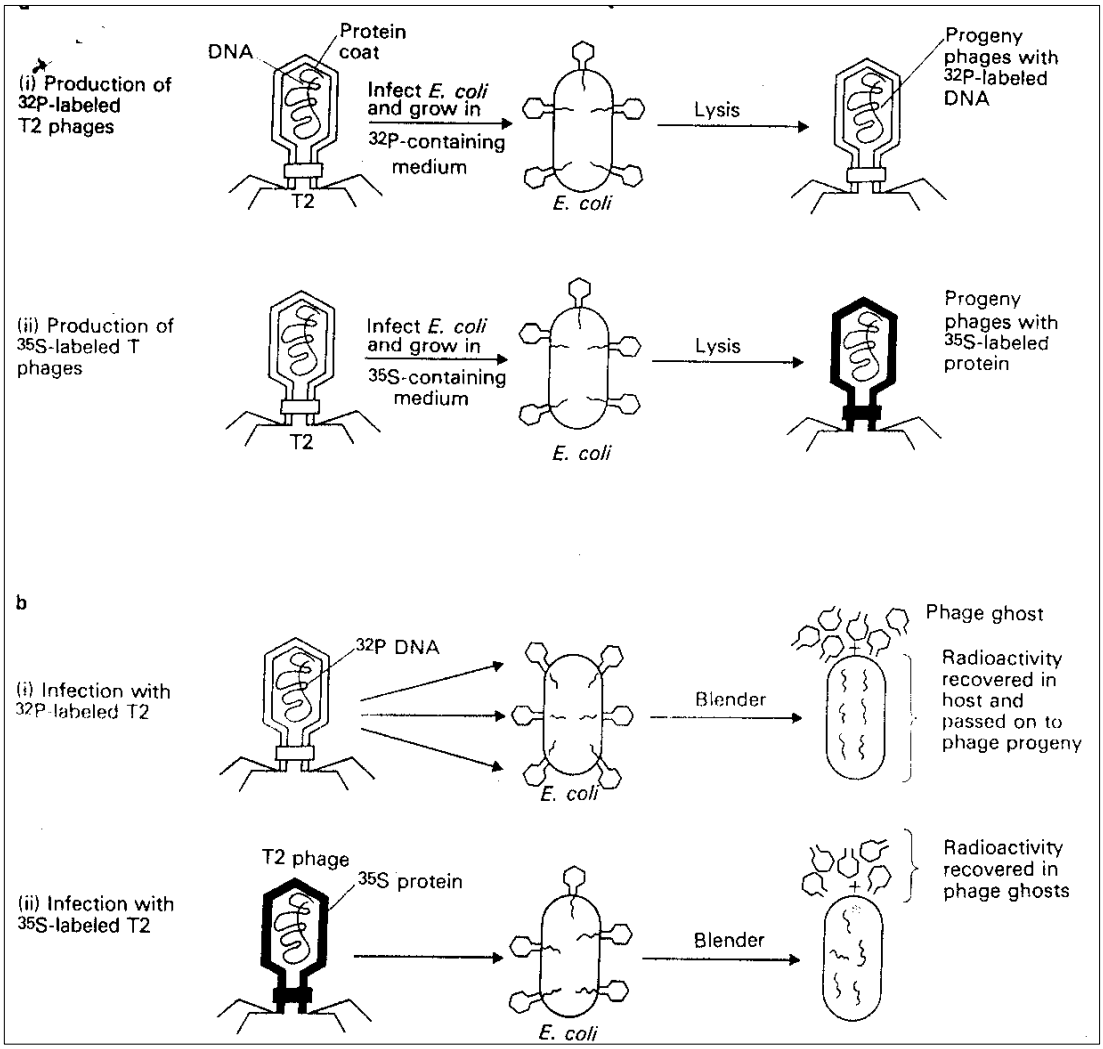

Fig. 1.3 The Hershey and Chase experiment. (a) Production of T2 phages either with (i) ^{32}P-labeled DNA or with (ii) ^{35}S-labeled protein, (b) Experiment that showed DNA and not protein was the genetic material of T2. In (i), *E. coli* infected with ^{32}P-labeled T2, and in (ii), *E. coli* is infected with ^{35}S-labeled T2.

In the next step of the experiment, unlabelled bacteria were infected with the labelled phage, and the distribution of the label in the host bacteria was then determined. When the infection was brought about by S^{35} labelled phage, little label was found within the host bacteria. Instead, most of the S^{35} labelled phage protein was found attached to the outside of the host bacteria, in the coats of the infecting viruses. When P^{32} labelled phages were used, little label was found in the protein coats and most of the label was found within the host bacteria. Therefore, it was principally the DNA that entered the bacteria during infection, whereas most of the protein remained attached to the outside of the bacteria. Thus the material injected into the bacteria by the virus is DNA, and it is this DNA that is necessary for the reproduction of genetically identical virus particles.

1.5 R.N.A. IS THE GENETIC MATERIAL IN SOME VIRUSES

All viruses contain protein and nucleic acid. Some viruses, like Tobacco Mosaic Virus (TMV) have RNA, rather than DNA, as their nucleic acid component. The experiments of Heinz Fraenkel-Conurat and Coworkers (1957) done towards the reconstitution of hybrid virus proved that the genetic information of TMV is stored in RNA, not in protein. The protein and RNA can be separated and the virus can be reconstituted by reassociating the components in a test tube. Reconstituted hybrid viruses can be produced by combining the RNA from one strain of TMV with the protein from another. When tobacco plants are infected with such a hybrid virus, the new virus produced in the plant is identical with virus of the strain that provided the RNA of the synthetic hybrids; characters of the strain that provided the protein does not appear. This showed that RNA carries the genetic specificity in TMV and some other viruses (Fig. 1.4).

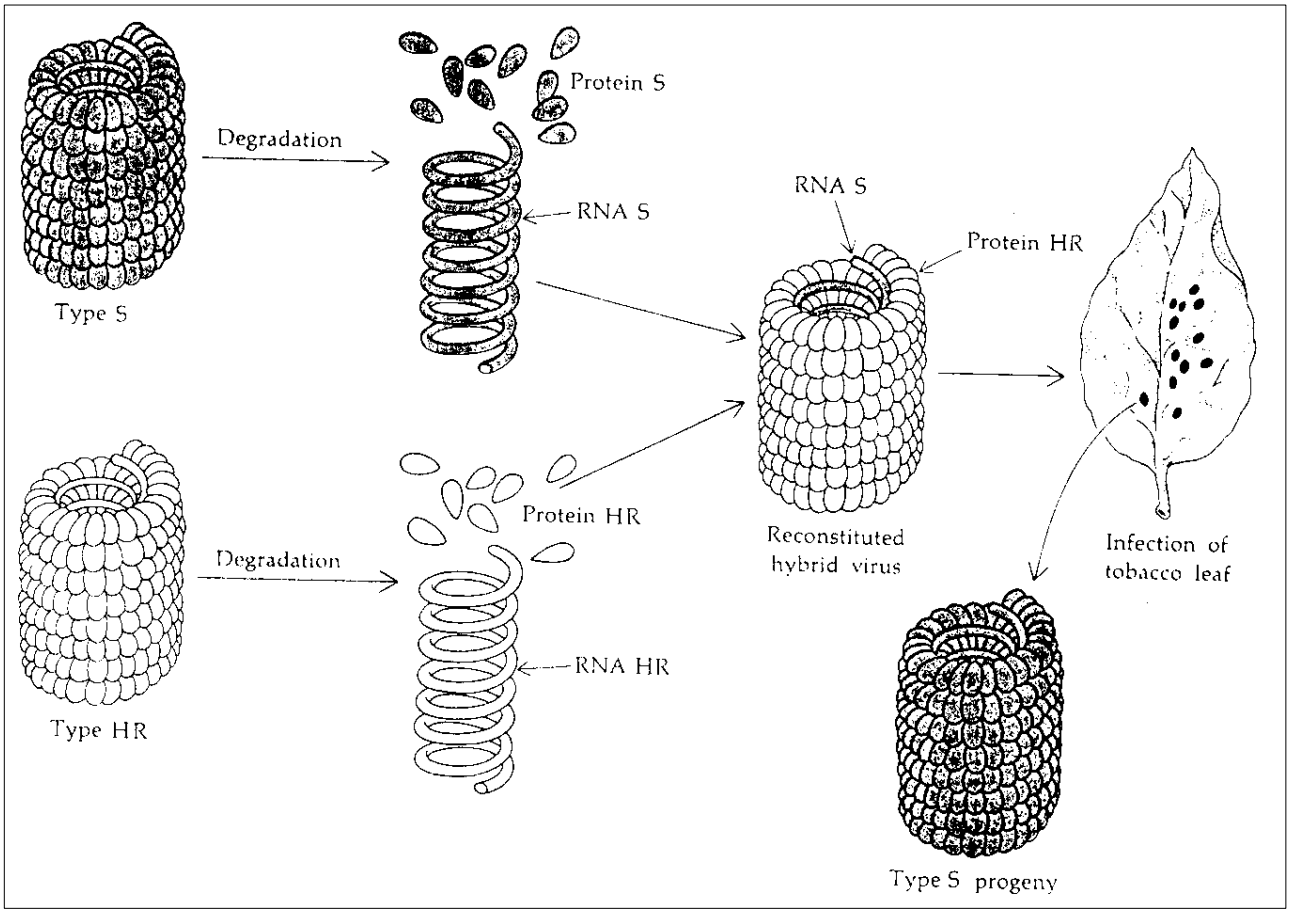

Fig. 1.4 Experiment to show that RNA is the genetic material in Tobacco Mosaic Virus (TMV). (From 'Modern Genetics' by F.J. Ayala and J.A. Kiger Jr., The Benjamin/Cummings Publ. Com. 1980). After separation of TMV particles into RNA and protein components, hybrid TMV particles can be reconstituted from the RNA of one strain (Type S) and the protein subunits of another strain (Type HR). Infection by the hybrid particles yields progeny consisting of both RNA and protein of the type specified by the RNA of the hybrid TMV.

1.6 D.N.A. IS THE GENETIC MATERIAL IN HIGHER ORGANISM AND IS LOCATED IN CHROMOSOMES

This was proved by the following experiments.

1.6.1 One of the chief characteristics of the genetic material is its constancy from generation to generation. Other macromolecules undergo continuous breakdown and resynthesis but DNA does not. Measurements (chemical analysis as well as photometry) of the amount of DNA per cell (expressed as 'C' value) of a given organism show that it remain essentially constant, whereas the amounts of other constituents vary from one cell type to another.

1.6.2 When eukaryotic cells were stained with basic fuchsin (called Feulgen reaction or Feulgen strain which is specific for DNA), the staining reaction occurs only in the chromosomes. If DNA is removed by treatment with DNAse or trichloroacetic acid, there is no visible Feulgen staining in the chromosomes.

1.6.3 The amount of DNA in a cell, though essentially constant, shows parallel changes with chromosome replications and division cycle. When the chromosomes duplicate, the amount of DNA doubles. After the cell has divided, each of the two daughter cells once again contain the original amount of DNA. This amount remains constant until the chromosomes duplicate in the next cell division.

1.6.4 By using special types of microscopes equipped with ultraviolet light source, the correspondence between UV absorption characteristics in chromosomes and pure DNA samples was demonstrated.

1.6.5 It was found that sperm of the liverwort *Sphaerocarpus donnellii* irradiated at 260 nm wavelength (maximum absorption by DNA) developed more hereditary changes than when irradiated at any other wavelength.

1.6.6 The extended thread like loops of lampbrush chromosomes of *Triturus* offered an opportunity to show that each strand contains DNA. Removal of protein (by protease) and RNA (by RNAse) did not effect the integrity of the loops in the chromosomes. However, DNAse treatment distorted the loop structure.

1.6.7 When Thymidine was synthesized using Tritium (radioactive isotope of hydrogen) and provided to the actively dividing eukaryotic cells, only chromosomes appeared to be radioactively labelled.

1.7 SUMMARY

Griffith working on *Streptococcus* bacteria showed for the first time that the genetic material from heat-killed Smooth bacteria is capable of changing or transforming living Rough bacteria. The experiments using cell free extracts by Avery, Macleod & McCarty, proved that the genetic material is of the nature of DNA. Hershey & Chases experiments on bacteriophage using radioactive sulphur and phosphorus demonstrated that DNA is the genetic material in phage. Experiments by Frankel conrat and colleagues with Tobacco Mosaic Virus showed that RNA is the genetic material in this virus. Experimental evidences are also available to confirm that eukaryotic chromosomes, which are known to be the hereditary carriers, that contain DNA. Thus, the chemical nature of the genetic material in majority of the organisms (all eukaryotes and many prokaryotes) was proved to be DNA. In some viruses, the genetic material is RNA.

1.8 MODEL QUESTIONS

i) Describe the experiments that proved DNA as the genetic material.

ii) Write short notes on:
   a) RNA as the genetic material in TMV
   b) Transformation
   c) Evidences to show that DNA is the genetic material in Eukaryotes
   d) Experiments by Hershey and Chase

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M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-III

Lesson 2

GENETICS OF BACTERIA - TRANSFORMATION, CONJUGATION & TRANSDUCTION

2.0 OBJECTIVES

This chapter describe, the methods of bacterial recombination namely, transformation, conjugation and transduction and their use in gene mapping.

2.1 INTRODUCTION

The survival of any species depends on its continued ability to remain adapted (live and reproduce) in its environment. This is largely determined by the size and diversity of the genetic pool of the population. There are important differences between crossing over in bacteria and in eukaryotes. Crossing over in eukaryotes occurs between paired chromosomes during meiosis I and results in an exchange of genetic material. The situation in bacteria is different. There is no meiotic process in bacteria. Many bacteria have evolved parasexual mechanism to enhance their genetic variability beyond the level possible through spontaneous mutations. The main mechanisms of genetic exchange in bacteria are transformation, conjugation and transduction which involve the

transfer of genetic information from one bacterium to another are described. These processes allow the propagation of many different genes and allels among the members of a population and thus enhance the gene pool for that population.

2.2 TRANSFORMATION

It is the movement of donor DNA across the cell membrane into the cytoplasm of recipient bacteria which is energy requiring process. Transformation does not occur in all cells, except competent cells, which secret a protein required for transformation.

A competent cell binds a large double stranded fragment of DNA at specific receptor site on the surface of the bacterium. Following binding, DNA moves across the cell membrane. One of the DNA strands is hydrolysed and degraded. The undegraded DNA strand is incorporated into the host chromosome (Fig. 2.1). This type of recombination is unidirectional and non-reciprocal.

Transformation and gene mapping

In mapping experiments using transformation, two genes are said to be linked, if they are frequently found on the same DNA fragment. If two bacterial genes are very close to each other, they will always be on the same DNA fragment, and thus cells have a high probability of becoming cotransformed with these genes e.g. his^+ and met^+. The farther apart two genes are, the greater the chance that they will not be found on the same fragrant. This is the basis of gene mapping experiments using transformation.

Two strains of bacteria are selected e.g. strain **A** is auxotrophic for amino acids his^{--} and met^{--} (they can't grow until they are supplemented with histidine and methionine in the minimal medium). Strain **B** is wild type for synthesis of amino acids his^+ and met^+. After a time for transformation to take place, B strain is tested for methionine and histidine properties.

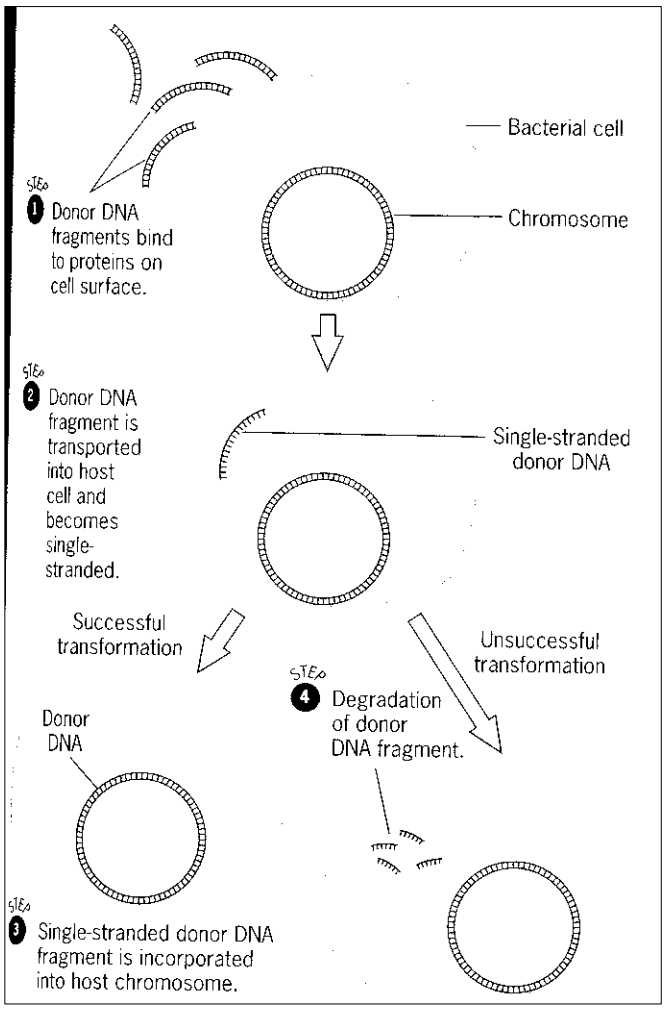

Fig. 2.1 Bacterial transformation →

Since we are interested in transformants the non-transformed cells can be eliminated by culturing on minimal medium with penicillin. The auxotrophs will not grow and hence will not be killed. The non-transformed prototrophs (his^+ and met^+) will be killed. By replica plating onto media, lacking histidine and methionine, it is possible to determine the phenotype of each transformed cell. Any transformation requires two crossovers (Fig. 2.2) of which one must be outside his-met region. The second crossover can be either on the other side of the region or between the loci. A ratio of single transformants to all transformants will measure the relative occurrence of cross over between the two loci, e.g. the following are numbers obtained in the experiment.

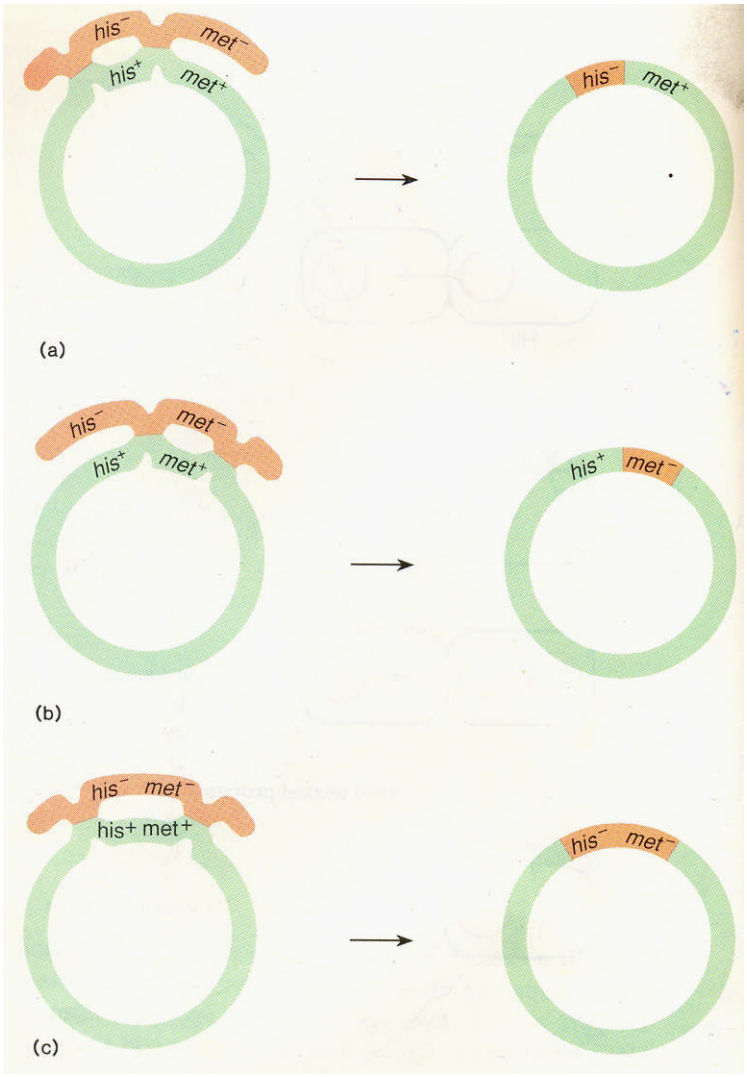

Fig. 2.2 Three crossover events that result in transformation in the experiment (a) and (b) only one locus is transformed because a crossover has occurred between the loci. In (c) both loci have been transformed because no crossover has occurred between them.

34 his met^{+} transformants
28 his^{+} met^{--} transformants
194 his^{---} met^{--} transformants

Then relative recombination frequency would be number of single transformants/total number of transformants =

$$\frac{(34 + 28)}{(34 + 28 + 194)} = 0.24$$

Thus we select all transformants and get relative occurrence of crossover between the loci in order to calculate a relative recombination index. Unlike mapping in eukaryotes, prokaryotic genetic work involves selection techniques since the selection techniques remove all cells in which no events takes place. Thus relative order of the genes can be obtained.

2.3 CONJUGATION

The conjugation is a process during which genetic information is transferred unidirectionally from a donor ("male") bacterium to a recipient ("female") through a cytoplasmic channel between the two cells. The required cell-to-cell contact between the donor and the recipient can be achieved through sex pili (e.g. *F. pili*) as in the case of enteric bacteria (e.g. *E. coli* and *Salmonella*) or through agglutinins (substances that promote cell clumping) or pheromones (chemicals produced by an individual that alter the behaviour of other members of the same species) as in the case of some Gram-positive cocci (e.g. *Enterococus faecalis*).

The discovery of parasexual process in bacteria by Lederberg and Tatum is from the study of auxotrophic strains (require some nutritional supplements in the medium). The auxotrophic strain A requires methionine and biotin for growth (Met^{--} Bio^{--}); a second strain B required threonine and leucin (Thr^{--} len^{--}) for growth. After plating the mixture of cells on a minimal medium, they discovered prototrophic colonies that could grow. These results suggested genetic recombination has occurred.

Davis proved through U-tube experiment that cell-cell contact was required for conjugation (Fig. 2.3). Hayes further discovered gene transfer was unidirectional.

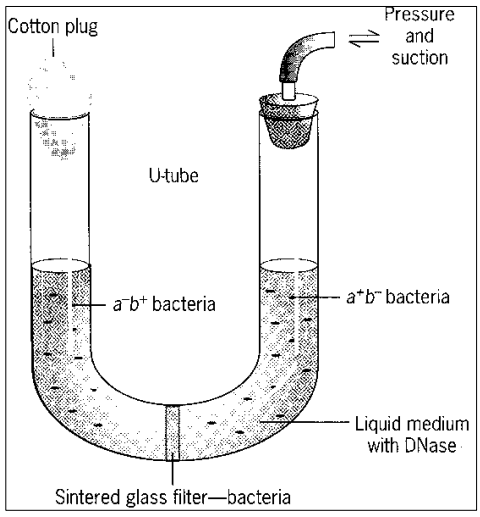

Fig. 2.3 The U-tube experiment. Bacteria of different genotypes are in different arms of the tube, separated by a sintered glass filter that prevents cell-cell contact. This experiment tests for conjugation because cell-cell contact is required for the transfer of genetic material. DNase in the

culture medium degrades free DNA, providing a test for transformation. If recombination occurs, it is likely taking place by means of transduction.

2.3.1 F^+ & F^- bacteria

The F^- factor (plasmid) of the bacterium, *E. coli* is a circular double-stranded DNA molecule. It measures 94.5 kilobase pairs (kbp) and is capable of directing its own (autonomous) DNA replication in the cytoplasm of the cell. The bacterial strains donate only plasmid genes from F factor during conjugation. These bacteria contain a "Fertility factor" that allows them to conjugate. The fertility is due to a type of plasmid known as a conjugative plasmid. It carries genetic information that codes for a protein called pilin, used to make a sex pilus known as the F pilus, as well as for proteins that promote the transfer of plasmid DNA from the donor to a recipient. F-cells that lack the F factor and thus are not pilated. Conjugation between an F^+ and an F^- bacterium begins with the attachment of the two cells via a sex pilus made by the F^+ cell. The sex pilus then retracts pulling the two cells into intimate contact with each other. The plasmid DNA then replicates by the rolling circle mechanism, only one stand of plasmid DNA is transferred from the donor to the recipient cell through the cytoplasmic bridge. The transferred single stranded DNA is replicated in the recipient cell producing double stranded molecule. When conjugation is complete, the exconjugant cells separate since each has atleast one copy of the plasmid, they both are now F^+ cells (Fig. 2.4).

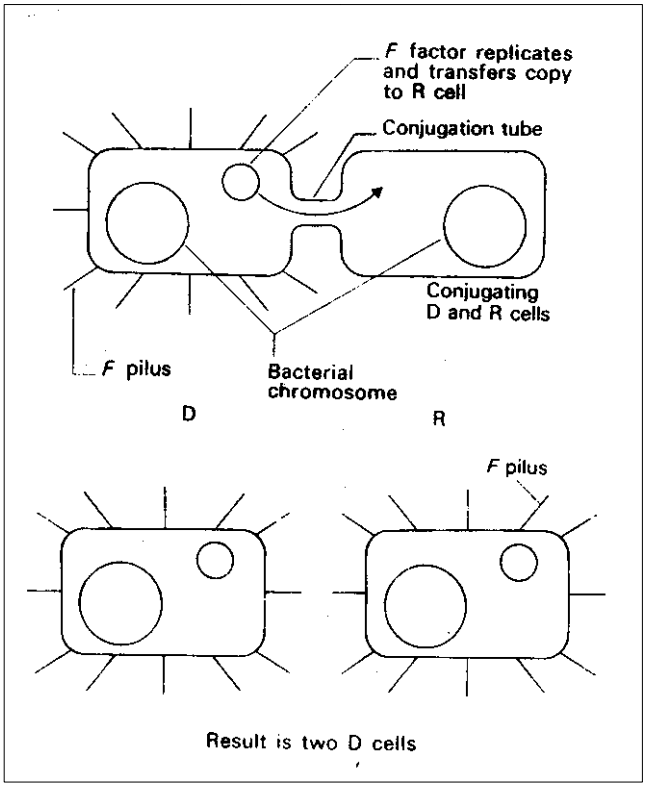

Fig. 2.4 Conjugation between F^+ (donor) and F^- (recipient) cells of *E. coli* results in transfer of a copy of the *F* factor and the conversion of the F^- to the F^+ state.

2.3.2 Hfr bacteria

Hfr strains were so named because they exhibit a high frequency of recombination for certain chromosomal genes. This high frequency of transfer by conjugation is due to the fact that the conjugative (F) plasmid has become incorporated into the bacterial chromosome (Fig. 2.5). The integration of the F plasmid into the bacterial chromosome takes place at several locations present on the chromsome. The regions or sites at which recombination occur are known as insertion sequences (IS) and consist of homologous DNA sequences between the conjugative plasmid and the chromosome. The process of DNA transfer between Hfr strains and F^- strains is essentially the same as that between F^+ and F^- strains. There is one significant difference, the recipient F^- bacterium always becomes F^+ after conjugation with an F^+ strain but it rarely becomes F^+ during conjugation with an Hfr strain.

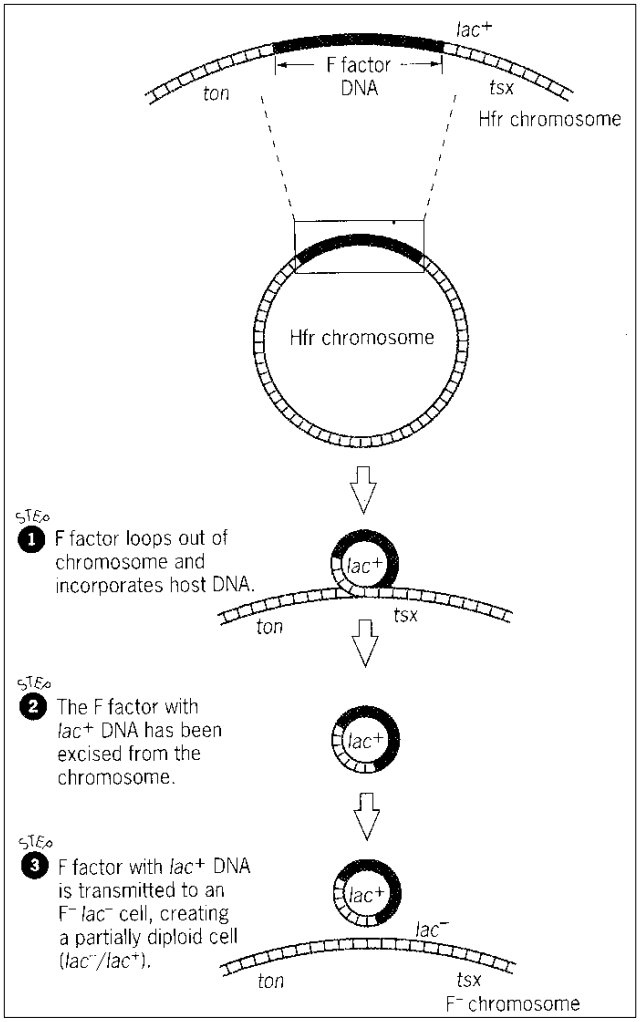

Fig. 2.5 Diagram of the formation of an *Hfr* strain by integration of the *F* factor, and reversion of the *Hfr* to the F^- state by the reverse process.

2.3.3 F^1 Conjugation or Sexduction

The integrated F factor in Hfr is excised from the bacterial chromosome, by reversing the steps that results in its integration. Sometimes there is error in the excision of the F factor from the chromosome (Fig. 2.5). This error results in the formation of an F′ factor - an F factor containing a segment of the bacterial chromosome. The bacterial genes incorporated in the F′ plasmid are transferred with very high frequency to recipient cells. The recipient acquires the F′ factor and becomes partially diploid for the bacterial genes that it carries. The transfer of bacterial genes by F′ factor is called F′duction or Sexduction.

2.3.4 Conjugation and Interrupted mating

In an interrupted mating experiment, conjugating cells are placed in a blender and broken apart. The Hfr x F^{--} mating is stopped at various intervals after the start. The first gene to enter the recipient is closest to the origin of transfer and the genes farther from the origin enter the recipient later (Fig. 2.6). In an Hfr x F^{--} conjugation experiment performed by Wollman and Jacob in 1950s, an Hfr strain that carried the genetic markers azi^{s} ton^{s} lac^{+} gal^{+} was mated to an F^{--} strain that was azi^{r} ton^{r} lac^{-} gal^{-} (the genes determine sodium azide resistance or sensitivity, phage T1 resistance or sensitivity and the ability to use lactose or galactose respectively as a carbon soruce. The **azi** gene was the first to enter the recipient, about nine minutes after the initiation of conjugation. The **gal** gene was the last to enter the recipient about 25 minutes after conjugation began. The **ton** and **lac** genes entered about 10 and 17 minutes respectively after the initiation of conjugation (Fig. 2.7)

The map generated by interrupted conjugation is circular and is marked off in units of time rather than crossover frequency. The large size of *E. coli* genome makes it impossible to map all the genes using a single Hfr strain. Hence, genetic maps are generated by using different Hfr strains with different F factor integration sites and different orientations. These maps are then combined to produce a final map of the *E. coli* chromosome (Fig. 2.8). The point is set at the thr locus because this is the gene closest to the point of insertion of the F factor first transcribed by Hayes.

2.4 TRANSDUCTION

Transduction is a mechanism of gene transfer in bacteria in which the donor DNA, is introduced into the recipient's cell via a bacterial virus (bacteriophage). During this process, new genetic information may be acquired by the host cell. There are two different types of transduction mechanisms. These are generalized transduction and specialized (restricted) transduction. Let us see, What is generalized transduction?

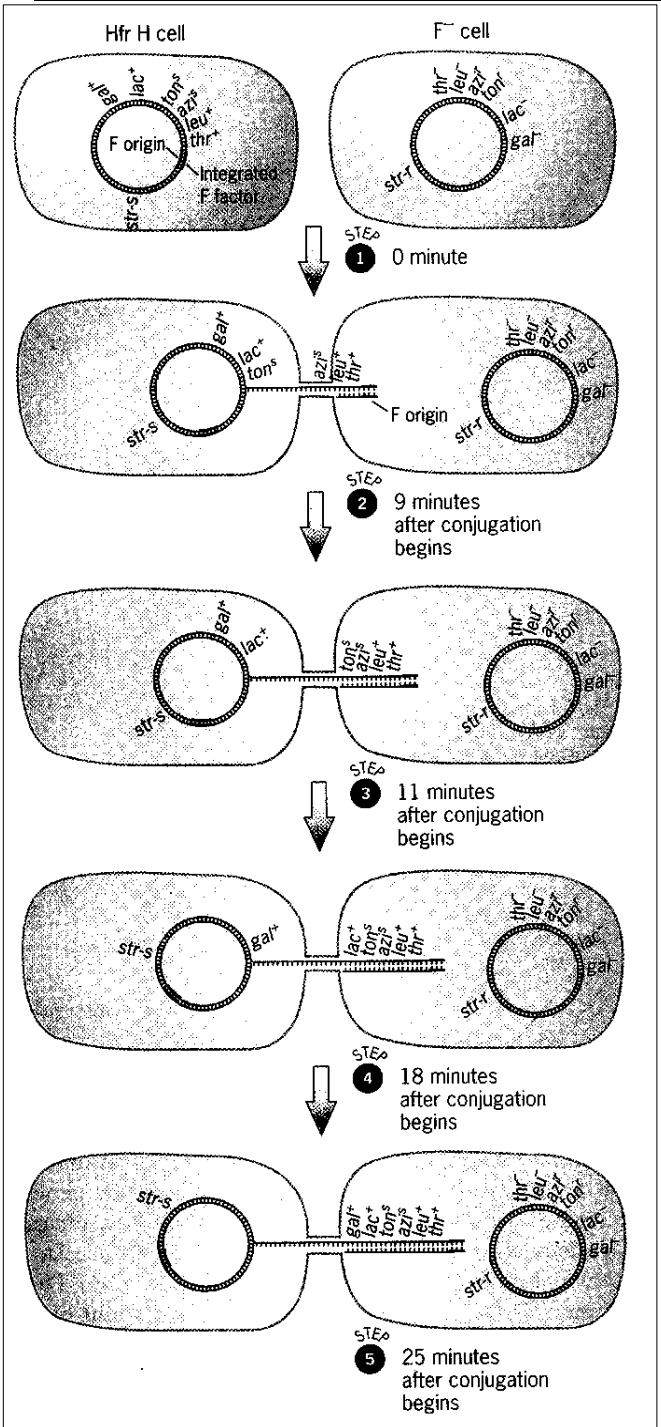

Fig. 2.6 The interrupted mating experiment of Wollman and Jacob. The transfer of the Hfr chromosome to the F⁻⁻ cell is linear, beginning at the origin on the F factor. Genes str-s (=*str*^{s}) and *str-r* (=*str*^{r}) confer streptomycin sensitivity and resistance, respectively, to the host bacterium.

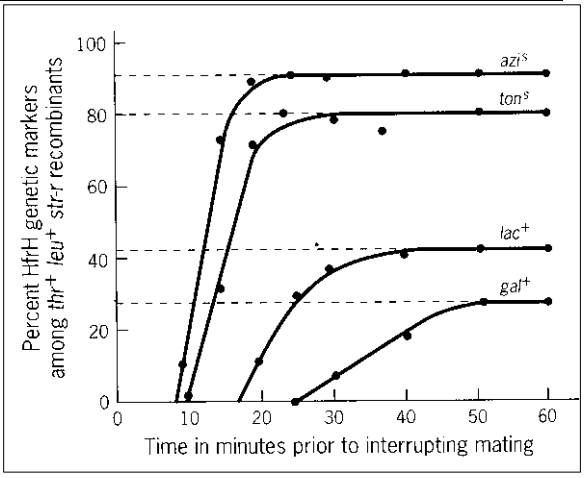

Fig. 2.7 Data from the interrupted mating experiment. The frequencies of the unselected donor markers in *thr*$^+$ *leu*$^+$ *str*^{r} recombinants are shown on the ordinate and time of entry on the abscissa. The dashed lines indicate the plateau frequencies observed for the various donor markers.

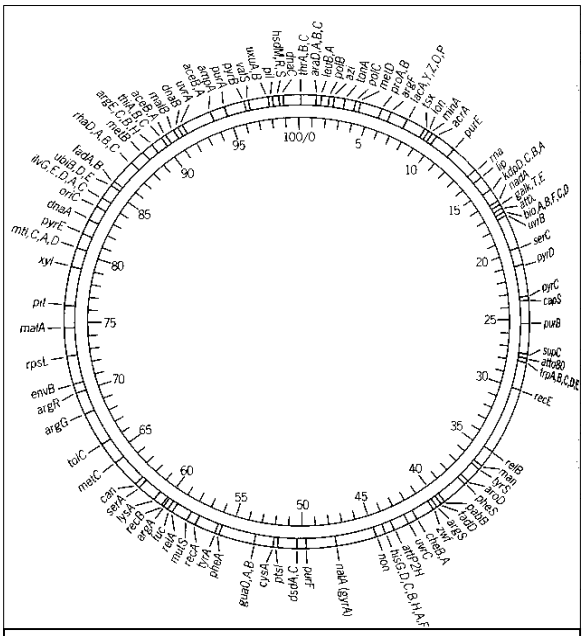

Fig. 2.8 A map of the *E. coli* genome, showing selected genes

2.4.1 Generalised transduction

Generalized transduction was discovered in 1952 by Lederberg and Zinder. During generalised transduction virtually any bacterial gene can be transferred (Fig. 2.9). The genetic transfer is mediated by a virulent or lytic bacteriophage. Lytic bacteriophage are bacterial viruses which upon infection of a host bacterial cell, destroy the DNA of the host and ultimately lyse the cell, releasing numerous viral progeny. The viral DNA is then replicated and viral proteins are synthesized. The newly replicated DNA is packaged into the coat proteins, and then infectious viral particles are assembled. When the viruses are fully assembled, viral enzymes degrade the cell's envelopes, lysing the cell and releasing the viral progeny.

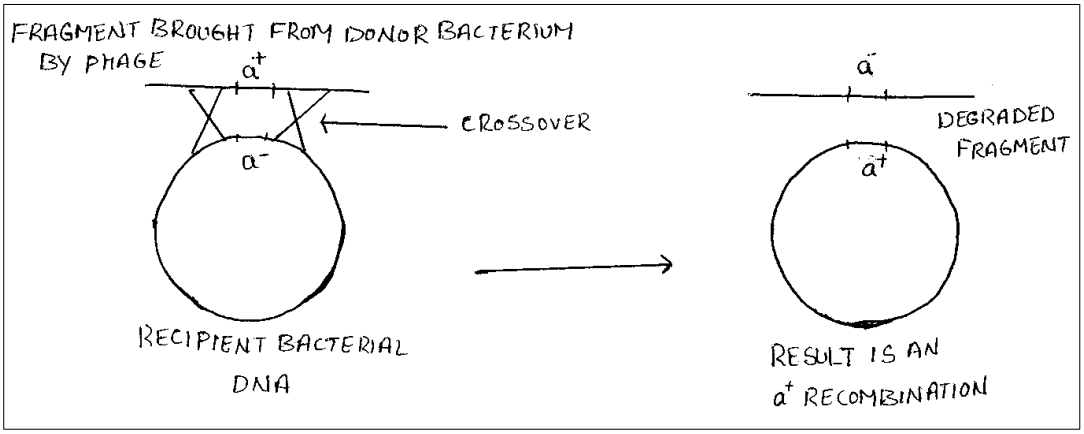

Fig. 2.9 Generalized transduction

Infrequently, however, some of the host's DNA is packaged into the virus along with an incomplete viral genome. When this happens, a generalized transducing phage is formed, which although capable of initiating an infection, is unable to replicate itself or lyse the host cell. These **defective transducing phages** serve as vehicles for the transfer of the host DNA (incorporated during viral assembly) from one cell to another (Fig. 2.9). Because the packing of host DNA into the viral particle is a random event, any given bacterial gene has an equal chance of being packaged and transferred to a recipient cell upon infection of a bacterial host cell by the transducing phage, the transducing DNA is introduced into the host's cytoplasm and becomes incorporated into the bacterial genome by homologous recombination. This cell is partially diploid for the introduced genes. Thus when bacterium divides, only one of the two daughter cells receives the donor fragment and express the donor genotype. The other cell, having lost the donor fragment reverts back to its original phenotype. This phenomenon is called **abortive transduction**.

2.4.2 Specialized transduction

In specialized transduction, the transducing particle carries viral DNA and specific portions of the bacterial genome. Specialized or restricted transduction is a process whereby a lysogenic bacteriophage (Phage λ) serves to transfer a specific gene at a high frequency. When lysogenic

bacteriophages infect host cells, its DNA is incorporated into the host's genome by recombination at a specific site (Fig. 2.10). The integrated λ genome is called a prophage Lambda (λ) unlike the T phages is a temperate phage, it has got two options, it can proceed through the lytic cycle like T phages destroying the host cell or it can enter into a lysogenic relationship with the host. In the lysogenic state, the DNA is replicated along with the host chromosome during the cell's replication cycle. However, the integrated λ is still able to cause cell lysis at later line. The prophage genome becomes excised from that of the host and undergoes a lytic life cycle, which results in the lysis of the host cell and the release of many phage particles.

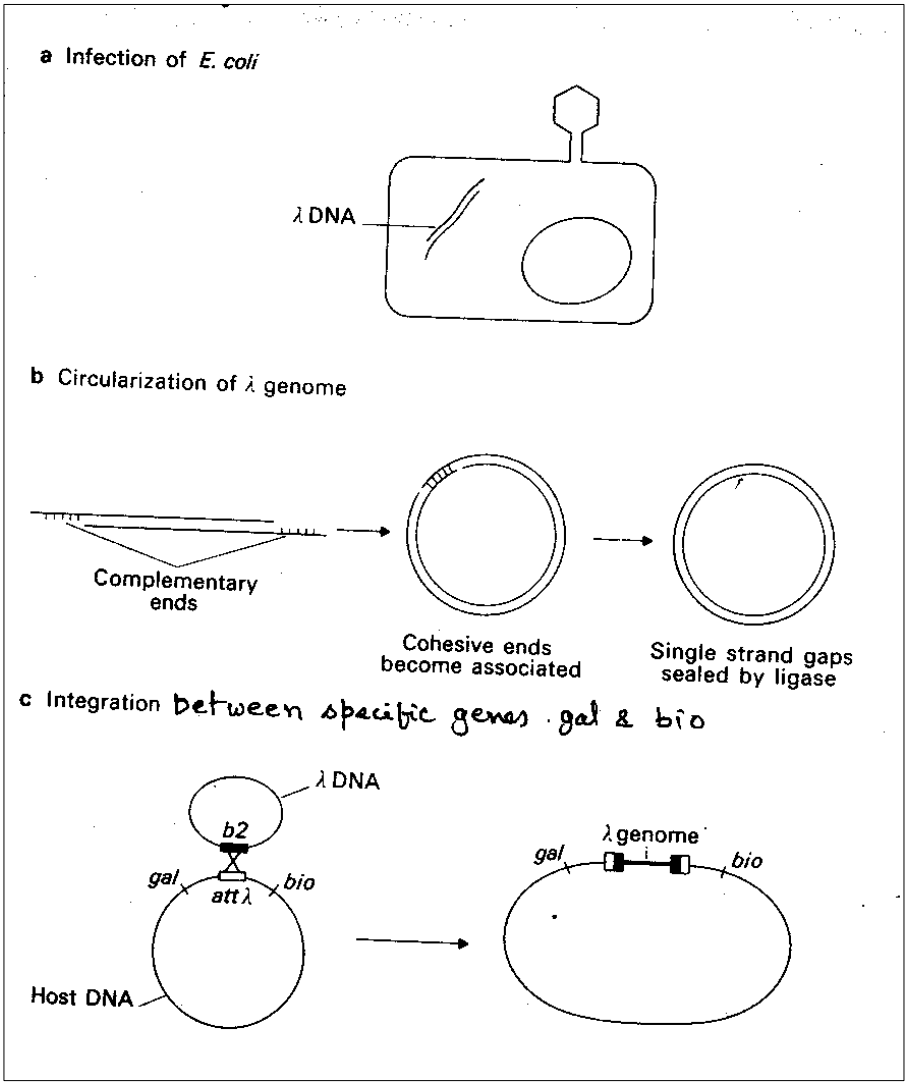

Fig. 2.10 Specialized Transduction

The site-specific recombination event of the lysogenic phage always occurs at a specified location and adjacent to certain genes [gal (galactose metabolism) and bio (biotin synthesis) genes].

Occasionally, the phage excision from the host's genome is defective and results in the release of a λ genome that contains part of the host's genome; also in particular, those genes (bio or gal) that are adjacent to the phage's site of insertion. Since the resulting, transducing bacteriophage is deficient in some of its genes, it can only serve to transfer the DNA to another host cell, but cannot go through a replicate cycle unless the cell also contains a wild-type phage. The transducting DNA becomes incorporated into the host's genome by site-specific recombination near the gal or bio locus. Specialized transduction can be used to map phage attachment sites and to analyze the genes that lie close to these sites. However, specialized transducing particles are not as useful in gene mapping experiments as generalized transducing phages.

2.5 SUMMARY

Transformation involves transfer of genetic material through direct (cell-free) absorption of DNA from the medium. Three process-conjugation, transformation and transduction, are used by bacteria to generate variability by transferring genes from an individual to the other. Conjugation involves such transfer of genes by establishing physical contact (cytoplasmic channel) between the donor and the recipient bacteria. The process of conjugation can be interrupted mechanically. The methods of interrupted mating technique in conjugation can be used to map the genes. The differences between F^+, F^- and Hfr strains, sexduction are described.

The process of transduction involves the transfer of genetic material from one bacterium to the other through a carrier, namely, a bacteriophage. Both transformation and transduction can be used to map the bacterial genomes through recombination mapping technique.

2.6 MODEL QUESTIONS

i) Describe the methods of gene transfer in bacteria.
ii) Explain the methods of gene mapping in bacteria.
iii) Write critical notes on
   a) Hfr
   b) Sex pili
   c) Interrupted conjugations
   d) Specialized transduction

2.7 REFERENCE BOOKS

i) **Essential Genetics** by P.J. Russell, Blackwell Scientific Publication, 1987.
ii) **Modern Genetics** by F.J. Ayala & J.A. Kiger Jr. The Benjamin/Cummings Publ. Com. 1980.

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A.U. Botany

M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-III

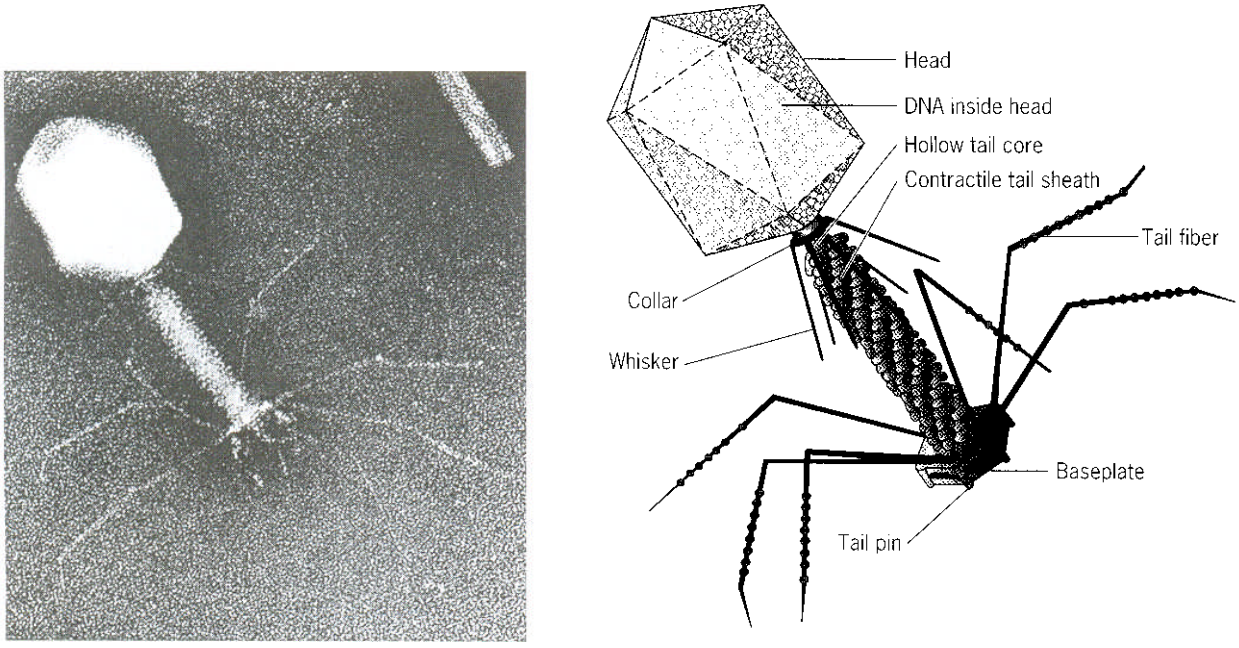
Lesson 3

Genetic Recombination in Phage - Fine structure of gene

3.1 INTRODUCTION

Viruses are ultramicroscopic intracellular parasites, capable of autonomous replication inside a host cell. Viruses were established as distinct from bacteria by the work of Frederick Twort and Felix D'Herelle during 1910-1917. D'He'relle called viruses, destroying bacteria as bacteriophages. Researchers began using bacteriophages as experimental organisms in 1930s to understand more about the genetic fine structure.

T phages, TI through T7 of *E. coli* are among the most intensely studied bacterial viruses. Though some structural variation occurs between T-phages, they are all tadpole-shaped (Fig. 3.1). The head is composed of several proteins, a polyhedron. Inside the head is a single molecule of double-stranded DNA. Attached to the head is a tail, composed of hollow tubes. The tail fibres uses to find a bacteria to infect. The tail pins anchor to virus to the host cell during infection.



(a) (b)

Fig. 3.1 Electron micrograph (a) and diagram (b) showin the structure of bacteriophage T4.

The structure of bacteriophage T4 is given (Fig. 3.1). When phage has absorbed to the bacterial cell surface, phage genetic material enters the bacterial cell. During the infection, the cells genetic material is destroyed, while the genetic material of the virus is replicated many times. New virus particles are assembled within the host cell, which bursts open releasing several hundred viral particles.

Certain phages are capable of replicating through different life-cycle stages lytic and lysogenic cycles.

3.2 PHAGE LIFE CYCLES

3.2.1 Lytic cycle

They replicate in the host cytoplasm and cause destruction of the host cell and liberate phage particles (Fig. 3.2).

3.2.2 Lysogenic cycle

These phages are capable of integrating into the host chromosome. Some temperate bacteriophages such as phage lambda, when infects *E. coli*, it may either cause lysis or become

lysogenic depending on the prevailing conditions. The integrated phage is termed a prophage (Fig. 3.2). Majority of research on lysogeny has been done on phage λ

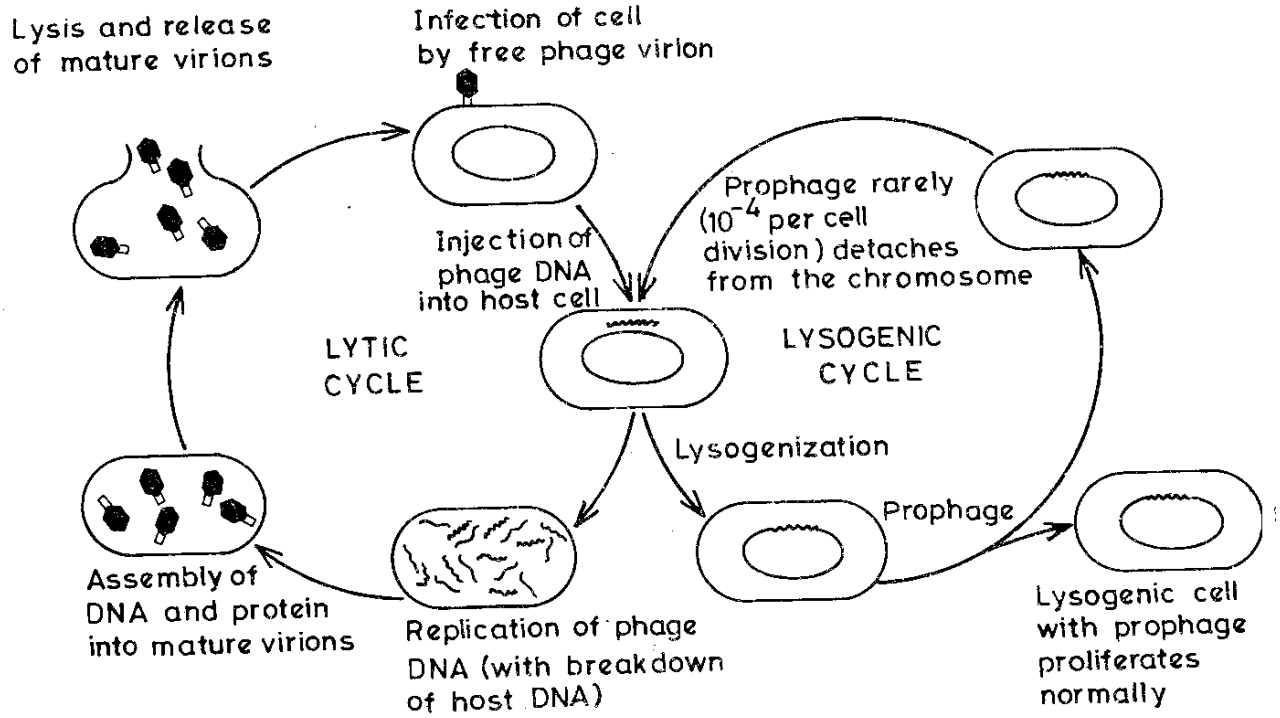


Fig. 3.2 The lytic and lysogenic cycles

3.4 PHAGE PHENOTYPES

Plaque morphology: It is a phenotypic trait used in phage genetics research. A plaque is a clear area in a lawn of bacterial cells that result from killing of contiguous cells by several cycles of bacteriophage growth. Plaques may be large or small with sharp or fuzzy edges (Fig. 3.3).

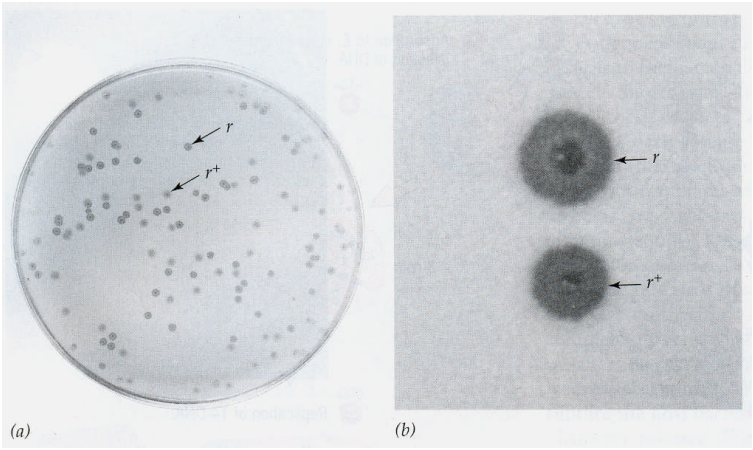


Fig. 3.3 (a) Wild-type (r^+) and rapid-lysis (R) mutant plaques of bacteriophage. T4 growing on a confluent lawn of *E. coli* strain B cells. (b) Higher magnification of individual r^+ and *r* plaques.

Host range: It is another frequently studied phenotypic trait in bacteriophages. Host range mutants are able to infect cells of some bacterial strains but not those of other strains. All wild type e.g. *E. coli* strain B cells can be infected by T-even bacteriophages. However, *E. coli*, B/2, a mutant *E. coli* is resistant to infection by T2 phage.

Genetic recombination in phage

In 1946, Max Delbrick and Alfred Hershey independently announced, the discovery of genetic recombination in phages. They simultaneously infected *E. coli* cells with two different strains of bacteriophage (Fig. 3.4) T2 – (1) h^+r (2) $h\ r^+$ i.e.

h^+ wild type phages
h - host range mutants
r^+ - wild type producing small plaques
r - rapid lysis mutants producing large plaques

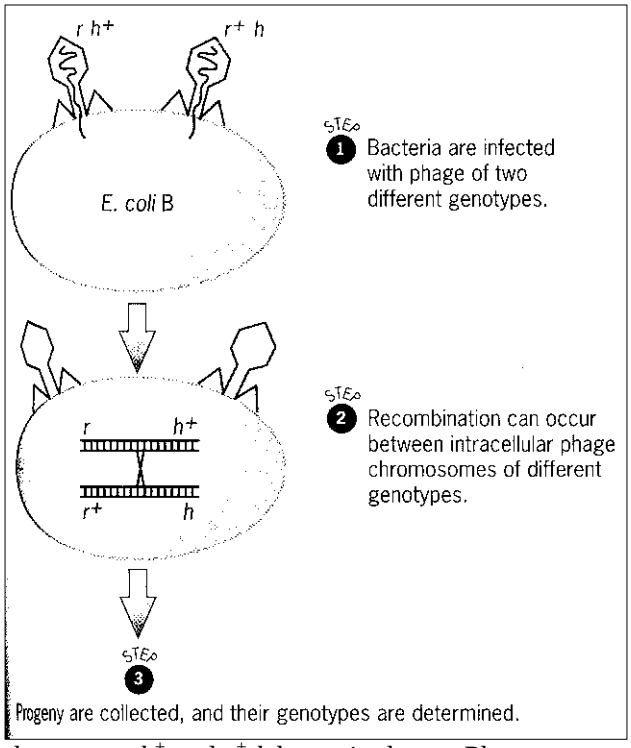


Fig. 3.4 A cross between *r* h^+ and r^+ *h* bacteriophage. Phage crosses are performed by simultaneously infecting bacteria with viruses of two different genotypes.

(1) They simultaneously infected *E. coli* cells with different strains of bacteriophage i.e. h^+r, hr.

(2) They collected progeny phage from this mixed infection.
(3) They produced a distinct plaque morphology when grown on these mixed lawns (Fig. 3.5).

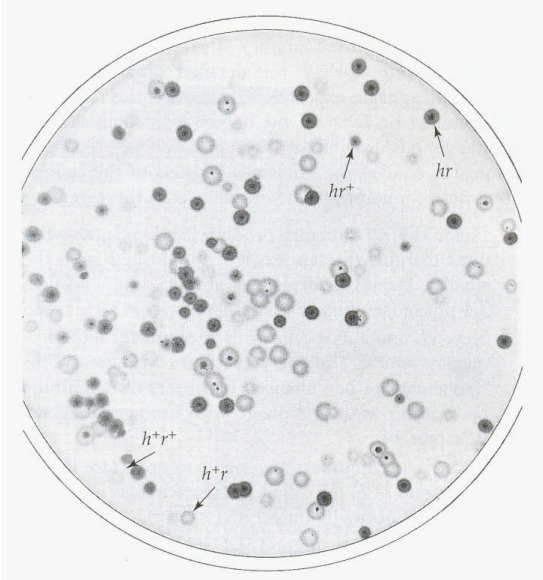


Fig. 3.5 Photograph showing the types of plaques formed by T2 *h r*, h^+ r^+, *h* r^+ , and h^+ *r* phage on a mixed lawn of *E. coli* B and B/2 cells

(4) Wild type (h^+ r^+) plaques were small and turbid, hr plaques were large and clear. They are parental genotypes.

(5) h^+r plaques were large and turbid; and h r^+ plaques were small and clear. These are recombinants.

(6) Hershey and Rotman found that 2 per cent of the progeny were recombinant and 98 per cent of the progeny were parental.

Parental types
h + r
h r^+

Recombinants
h^+r^+
h r

A single cross over in this region will produce the recombinants. The percentage of recombination is the map distance, which is a relative index of distance between loci. The greater the physical

distance, the greater the amount of recombination. The discovery of recombination allowed them to estimate that the distance between h and r genes was about 2 map units.

Two important points emerged from the early phage mapping studies.

1. Recombination appeared to occur by a reciprocal process as occurred during meiosis in eukaryotic organisms. If one recombinant class was present among the progeny produced by a population of doubly infected bacteria, the other recombinant class was present at approximately the same frequency.

2. Recombination in phages is not the same as meiotic recombination. In meiosis, chromosomes synapse and cross over at a specific stage. In viruses there is no process as complicated as meiosis. Recombination can occur at any time during the phage life cycle as long as DNA has not been packaged into protein coats. Multiple rounds of genetic exchange can occur.

3.5 GENE FINE STRUCTURE

Gene mapping took an entirely new dimension in mid 1950s, when Seymour Benzer mapped 2400 r II mutants to 304 sites separable by recombination in two contiguous genes in a small region of the bacteriophage T4 genome. His experiments extended the results of earlier studies on *Drosophila,* which showed that the gene is divisible by both mutation and recombination. Benzer studied r II genes because of the unique properties of mutant phage with defects in these genes.

1. Phage T4 r II mutants produce large plaques with clear margins, easily distinguished from the smaller , fuzzy-edged plaques, produced by wild type T4 phage.

2. New r II mutants occur with a relatively high frequency – about in every $100,000^{r+}$ progeny.

3. Phage T4 r II mutants are unable to grow on a strain of *E. coli* called K12 (λ), whereas wild type T4 grows normally on K12 (λ).

4. Wild type T4 phage and T4 r II mutants are both able to grow on *E. coli* strain B cells.

Benzer utilized the system in the analysis of r II region by crossing two independently occurring r II mutants on *E. coli* B cells (Fig. 3.6), then collecting the progeny. If recombination occurred between the two mutants, a wild type chromosome would result, along with a double mutant (Fig. 3.6). A major advantage of r II system is, the recombination frequencies as low as 10^{-6} could have

been detected in Benzer's experiments. The r II mutants are conditional lethal mutants i.e. wild type T4 phage can grow on both *E. coli* B and K12 (λ), r II mutants can grow only on *E. coli* strain B.

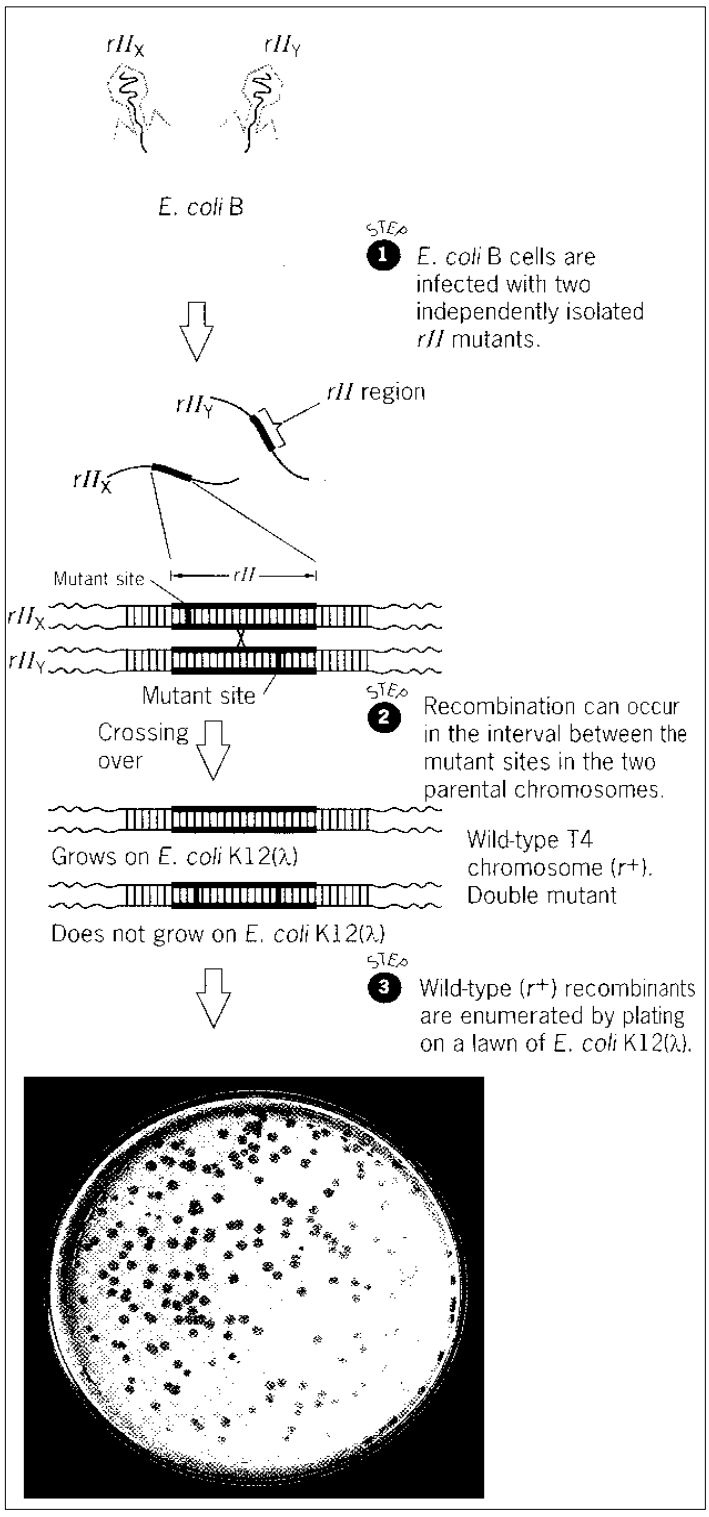


Fig. 3.6 A genetic cross in which **E. coli** B cells are infected with two independently isolated phage Tr r II mutants. The recombinant progeny are wild-type (*r*+) and double mutants.

If progeny phage produced from a mixed infection of *E. coli* B cells are plated on laws of K12 (λ) cells, only wild type T_4 phage can form plaques. If 20 plaques form on K12 (λ) lawn, 20 wild type recombinant phages are present. However, if recombination is a reciprocal process, one double mutant recombinant will be present for each wild type produced by recombination. The 20 wild types are produced by recombination. The 20 wild type plaques indicate that 40 recombinant phages are present. Thus recombination frequencies and map distances are calculated a follows:

$$\text{Recombination frequency} = \frac{2 \times \text{number of wild-type recombinants}}{\text{total number of progeny}}$$

and

$$\text{map distance (\% recombinants)} = \frac{2 \times \text{number of plaques on K12 } (\lambda)}{\text{number of plaques on B}} \times 100$$

To estimate the frequency of recombination, the total number of progeny phage produced per unit volume of mixed infection lysate must be determined. All the progeny phage produced in a cross between two r II mutants can grow on *E. coli* B cells. If 100 plaques are counted in a sample diluted 10^6 fold, the original sample contained 10^8 phage per unit volume (100×10^6). Thus, if 40 recombinant phage (wild type and double mutant) are present among 10^8 total progeny, the recombination frequency between the two mutant sites will be $40/10^8 = 0.0000004$

These calculations overlook only one fact, if an r II mutant may undergo spontaneous mutation back to wild type (r^+). However, such reversion rate of mutations are usually negligible and therefore, the above calculations are considered.

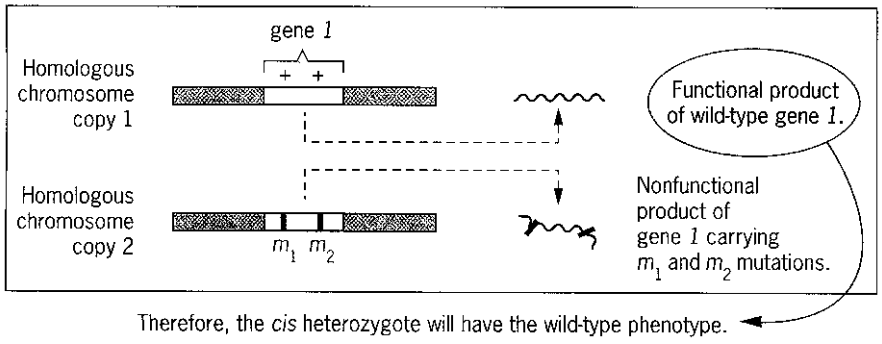
All of Benzer's r II mutants contained mutations in one of two contiguous genes, designated r II A and r II B. He determined this by performing complementation test.

3.5.1 Complementation test – Operational definition of the gene

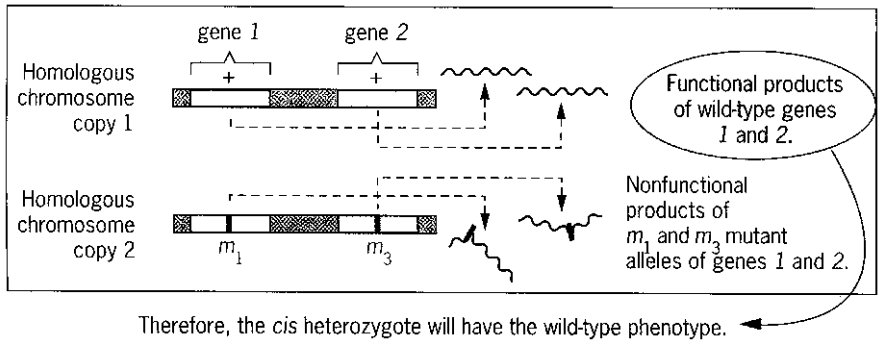
A double heterozygote, carries two mutations and their wild type alleles i.e., $\mathbf{m_1}$ and $\mathbf{m^+}$ **plus** $\mathbf{m_2}$ **and** $\mathbf{m_2^+}$**.** They can exist in two arrangements. When two mutations are on the same chromosome, the arrangement is called the **coupling** or **cis configuration**; a heterozygote with this genotype is called **cis heterozygote**. When two mutations are on different chromosomes, the arrangement is called the repulsion or **trans configuration**. An organism with this genotype is a **trans-heterozygote**.

The complementation test allows geneticists to determine whether mutations that produce the same or similar phenotypes are in the same gene or in different genes, complementation test is

referred as the **cis-trans test**. Each **cis heterozygote** which contains one wild type chromosome, should have wild type phenotype, whether the mutations are in the same gene or in two different genes (Fig. 3.7). If the ***trans*** **heterozygote** has the mutant phenotype then the two mutations are in the same unit of function, the same gene (Fig. 3.8a). When a ***trans*** **heterozygote** has the wild type phenotype, the two mutations are said to exhibit complementation or to complement each other and are located in two different units of function, two different genes (Fig. 3.8b).



(a) *cis* heterozygote: mutations in one gene.

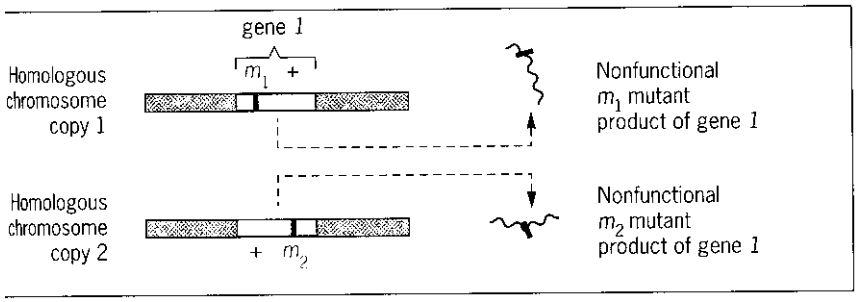


(b) *cis* heterozygote: mutations in two different genes.

Fig. 3.7 The *cis* test. The *cis* heterozygote should have the wild-type phenotype whether the mutations are in the same gene (a) or in two different genes (b).

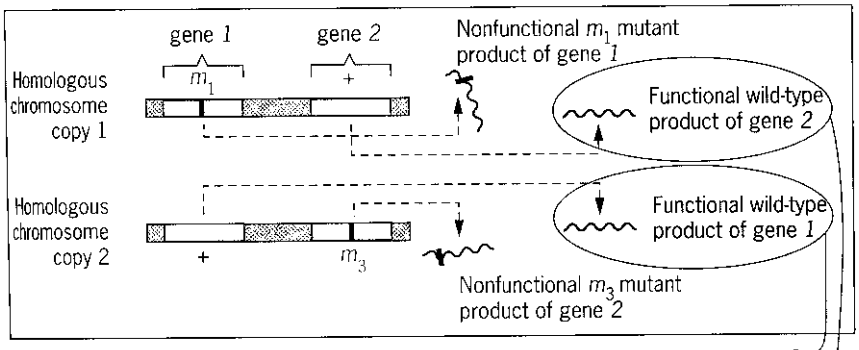
All of Benzer's r II mutants contained mutations in one of two contiguous genes, designated r II A and r II B. He determined this fact by performing complementation test.

Benzer infected *E. coli* K12 (λ) cells with a pair of r II mutants. If the two r II mutants contained mutations in the same gene, they failed to grow on *E. coli* K 12 (λ), if they were mutant in different genes (one was r II A and other was r II B) they grew on *E. coli* K12 (λ). Benzer characterized a number of r II mutants by the following procedure.



No functional gene *1* product is synthesized in the *trans* heterozygote; therefore, it will have a mutant phenotype.

(*a*) *trans* heterozygote: mutations in one gene.



Functional products of both genes are synthesized in the *trans* heterozygote; therefore, it will have the wild-type phenotype.

(*b*) *trans* heterozygote: mutations in two different genes.

Fig. 3.8 The *trans* test. The *trans* heterozygote should have (a) the mutant phenotype if the two mutations are in the same gene, and (b) the wild-type phenotype if the mutations are in two different genes.

3.5.2 Deletion mapping

After mapping about 60 mutations by two-factor crosses, Benzer devised a new, more efficient procedure, to map the rest of 2400 r II mutants. Benzer's short cut procedure is called **deletion mapping**. He demonstrated that some of his r II mutants contained deletions of all or part of r II region. A phage that carries a deletion cannot mutate back to wild, nor it recombine with another phage. Hence, a deletion mutant can't produce wild phenotype. Therefore, any r II mutant that produce wild type recombinants carry a mutation that maps outside the deleted segment, and any mutant that fails to generate wild-type recombinants, must carry a mutation that maps within the area defined by the deletion.

Benzer accomplished this by crossing different deletion mutants (Fig. 3.9). The appearance of wild-type recombinant progeny indicate that the deletions did not overlap using deletion mapping, Benzer estimated the extent of the deletions.

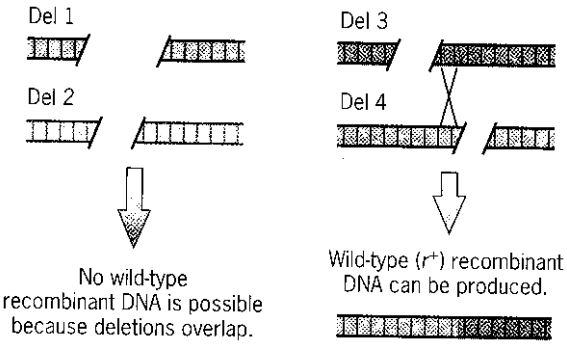


Fig. 3.9 Crosses between pairs of deletion mutants. If the deletions overlap, no wild-type recombinants are produced, if they do not overlap, *r*+ progeny are produced.

Benzer identified seven large deletions, that were missing overlapping segments of the r II locus containing r II A and r II B genes.

Benzer also isolated and characterized many smaller deletions that defined 47 short intervals within the r II region (Fig. 3.10). Benzer used this procedure to map 2400 r II mutants within r II A and r II B genes. Four important conclusions emerged from Benzer's analysis of the r II region.

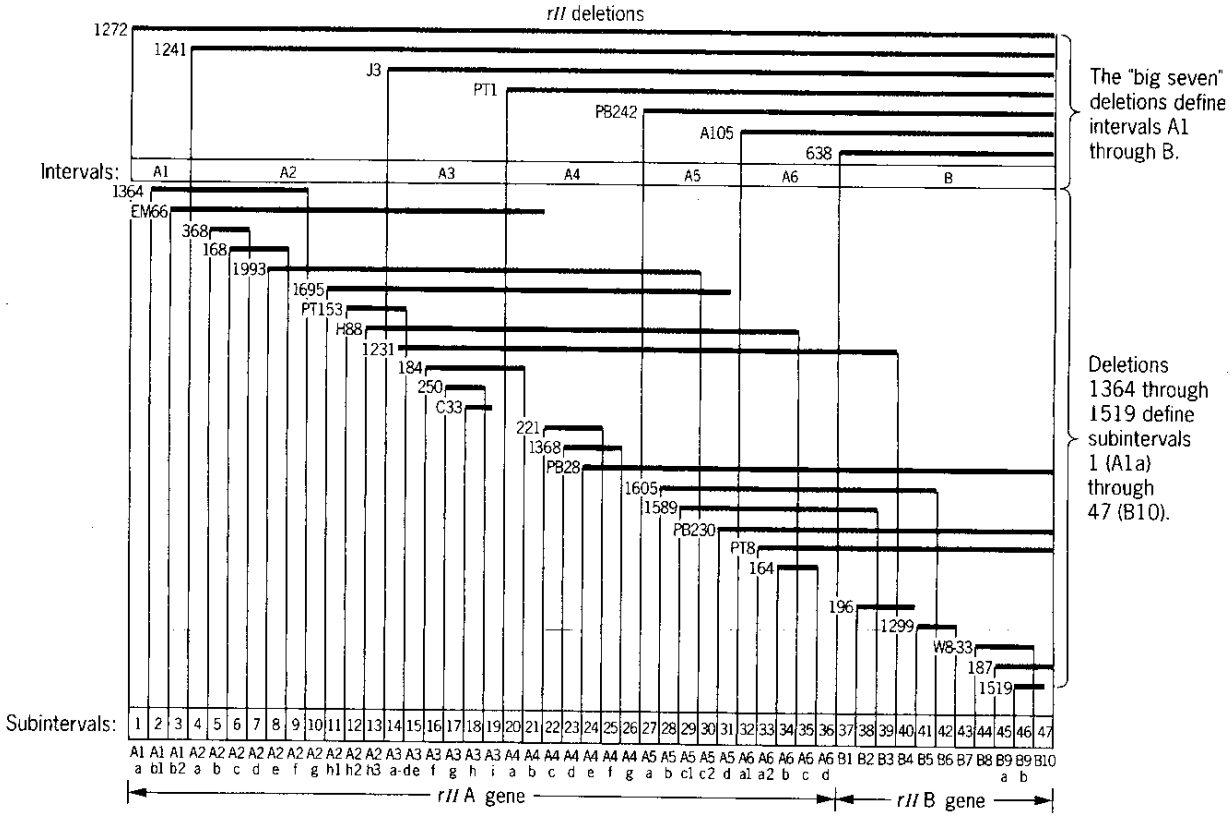


Fig. 3.10 The extent of the deletions carried by the deletion mutants used by Benzer to construct his fine structure map of the *r II* locus of bacteriophage T4.

(1) Since 2400 *r II* mutants mapped to 304 different sites are separable by recombination, the mutants were not randomly distributed over the 304 sites. Some r II sites are called mutation **hot spots** since 500 spontaneous mutations occurred at one hot spot in r II B gene (Fig. 3.11).

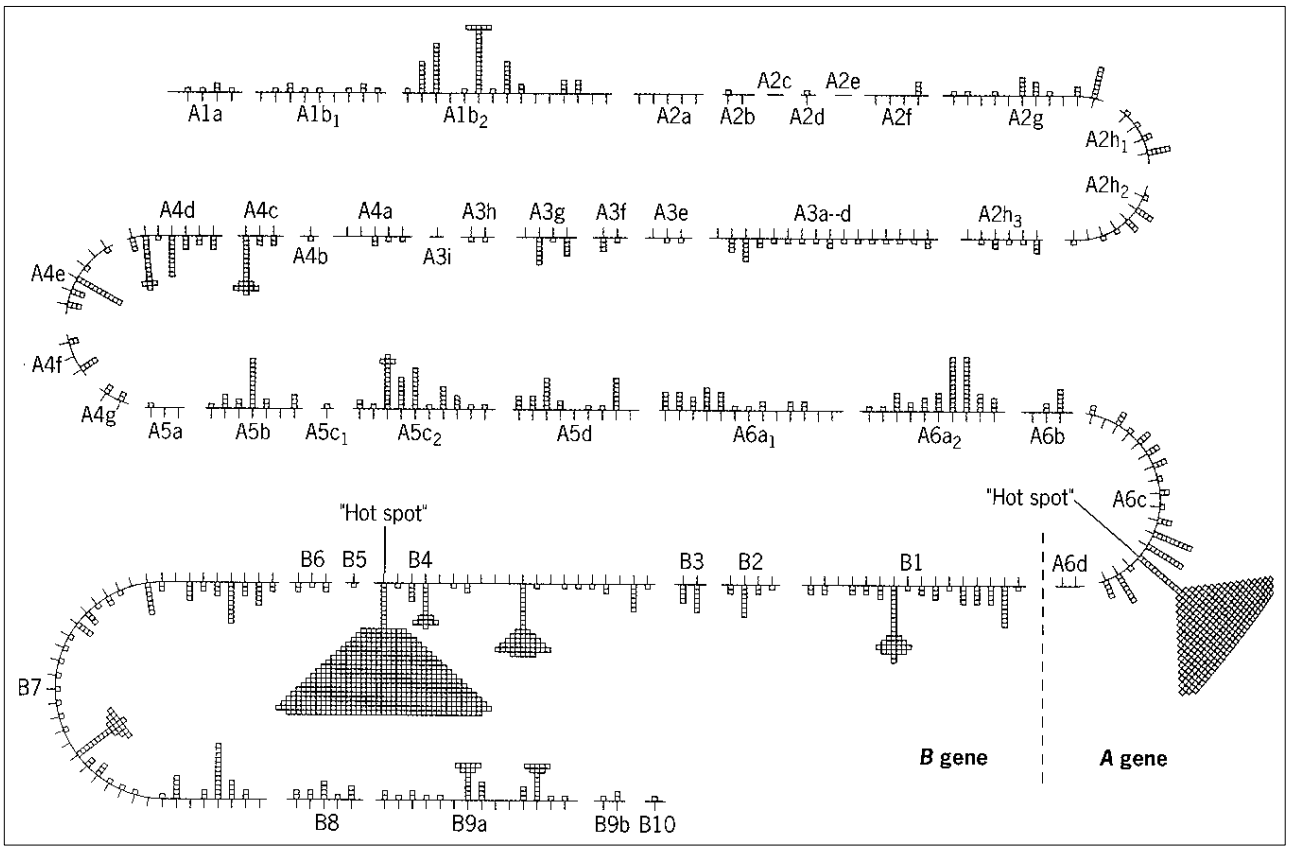


Fig. 3.11 The genetic map of the *r II* locus of phage T4. The *r II* locus contains two genes, *r II A* and *r II B*. Each square represents the independent occurrence of a spontaneous mutation at the indicated site. Vertical lines at which no squares occur represent positions defined by mutations induced by mutagenic agents. Some sites, called hot spots, mutate at very high frequencies.

(2) All 2400 r II mutants were mapped to either r II A gene or r II B gene or both genes.

(3) The smallest recombination frequency that Benzer observed was 0.02 per cent. The complete T_4 map is about 1500 map units length, this frequency represents 0.02/1500 = 0.00133 per cent of the map given that the T_4 genome is 173,000 base pairs in length, the smallest interval in which recombination can occur is (0.0000133) x 173,000 or 2.3 base pairs. Thus Benzer's results suggested that recombination can occur between any pair of adjacent nucleotides.

(4) Benzer's work extended the results of earlier experiments with *Drosophila* demonstrating that the gene is divisible by mutation and recombination.

The existence of a detailed map of *r II* locus of phage T_4 provided researchers, more opportunities to understand the fine structure of gene, insights about recombination mechanisms. Indeed Benzer's analysis of T_4 *r II* locus stands today as one of the great achievements in understanding modern gene concept.

3.7 SUMMARY

Genetic recombination in phages is understood through Benzers analysis of *r II* mutants. The analysis of mutants by deletion mapping and the frequency of recombination between mutant sites led to the understanding of fine structure of the gene. Hence, gene as a divisible unit of function **cistron**, unit of recombination **recon** and unit of mutation-**muton** is described.

3.8 MODEL QUESTIONS

1. Give a brief account of process of Genetic recombination in phages.
2. Discuss Benzer's work that have led to the understanding of fine structure of a gene.
3. Write notes on:
   a) Deletion mapping
   b) Lysogenic cycle
   c) Cis-trans test

3.9 REFERENCE BOOKS

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Prof. T.N. MARY

M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-III

Lesson 4

Evolution of Gene concept

4.1 INTRODUCTION

The concept of the gene has undergone many refinements since the rediscovery of Mendel's work in 1900. The gene is the basic unit of genetic information. In this chapter, I have described how the concept of the gene changed since it Mendel's work during 1900 to the present day.

IMPORTANT MILESTONES IN GENETICS AND GENOMICS*

Year	Discovery	Scientists
1859	Origin of species	Charles Darwin
1865	Laws of segregation	Gregor Johann Mendel
1869	Isolated DNA	Friedrich Meischer
1879	Walter Flemming	Chromosomes
1900	Rediscovery of Mendelian laws	DeVries, Correns & Tschermak
1902	In-born errors of metabolism	Archibald Garrod
1902	Genes on chromosome	Walter Sutton & Theodor Boveri
1905	Coining of the term Genetics	Willian Bateson

Year	**Discovery**	**Scientists**
1908	Hardy-Weinberg law	Hardy & Weinberg
1909	Hybrid vigour	Shull G.H.
1909	Coining of the term gene	Wilhelm Johannsen
1911	Sex-linked gene in *Drosophila*	Thomas Hunt Morgan
1913	Genetic linkage maps	Alfred Sturtevant
1927	X-rays as a mutagen	Hermann Muller
1928	Transforming principle	Griffith
1941	One gene – one enzyme hypothesis	Beadle & Tatum
1944	DNA as a genetic material	Avery, Macleod & McCarty
1946	Conjugation in bacteria	Lederberg & Tatum
1952	Jumping genes	Barbara McClintock
1952	Genes made of DNA	Alfred Hershey & Martha Chase
1953	Double helical structure of DNA	James D Watson & F.H.C. Crick
1958	Semi-conservative DNA replication	Mathew Messelson & Franklin Stahl
1958	DNA polymerase	Arthur Komberg
1961	*Lac Operon*	Jacob & Monod
1966	Dwarfing genes	Norman E Borlaug
1966	Genetic code	Har Gobind Khorana
1966	Central dogma	Francis H.C. Crick
1970	Restriction enzyme *Hindll*	Hamilton Smith
1972	Recombinant DNA	Paul Berg & Cohen
1975	Southern transfer	Edward Southern
1976	Reverse transcriptase	Baltimore & Thame
1976	Genetech company	Herbert Boyer
1977	DNA sequencing	Sanger
1977	Split genes with introns	Phil Sharp & Richard Roberts
1977	ϕX-174 genome sequenced	Sanger *et al.*
1980	RFLP map of human genome	Botstein *et al*.
1986	Polymerase chian reaction	Kary Mullis
1987	Automatic DNA sequencer	Leody Hood
1987	Mapmaker software program	Erick Lander
1990	Human Genome Project launched	Watson *et al*.
1990	BLAST software	Altschul & others
1995	*H. influenzae* genome sequenced	Venter *et al*.
1996	Yeast genome sequenced	International consortium
1997	*E. coli* genome sequenced	Blattner *et al*.
1998	*C. elegans* genome sequenced	Goffeau *et al*.
1999	Human chromosome 22 sequenced	Dunhan *et al*.

2000	*Drosophila* genome sequenced	Venter *et al*.

Year	Discovery	Scientists
2000	Human chromosome 21 sequenced	Hattori *et al*.
2000	*Arabidopsis* genome sequenced	International consortium
2000	*Oryza sativa* genome sequenced	Monsanto & Syngenta Pvt. Ltd.
2001	Entire human genome sequenced	Venter *et al*.

*Information available in http://www.netscape.org/Mendel Web/Mwtoc.html and http://gnn.tigr.org/timeline/timeline_frames.shtml.

4.2 REDISCOVERY OF MENDEL'S WORK

In 1866, Mendel discovered **unit factors**, which are now called genes that controlled one specific phenotype e.g. flower colour, seed shape etc. (Fig. 4.1). He discovered that traits are not inherited as such, **units** or **factors** determine the observable characteristics which are transmitted from parents to offspring. Mendel's classic paper did not become an accepted part of scientific knowledge until after the rediscovery of Mendel work in 1900. The pathway by which a gene exerts its effect on the phenotype of an organism is often very complex (Fig. 4.2). Ultimately each gene has a potential effect on the phenotype of an organism or the population or the species to compete for an ecological niche in the biosphere.

The definition of the gene as the basic unit of function is accepted by most geneticists. However, the elucidation of the chemical nature of the genetic material raised questions about the structure of the gene and our understanding of the molecular structure of this basic unit of function, which has undergone several refinements since the discovery of Mendel's work in 1900.

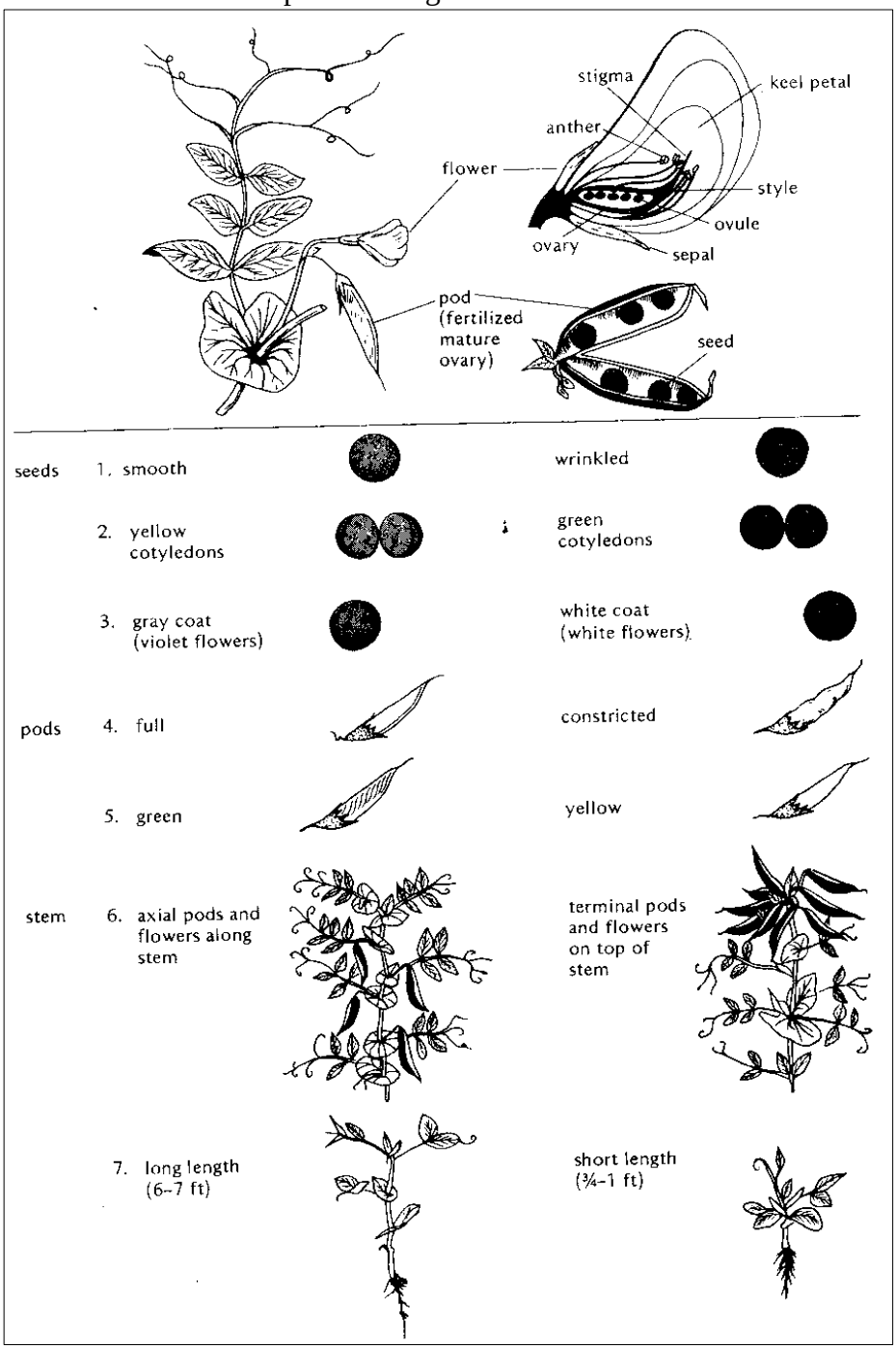
Bateson (1906) and Morgan (1912) made extensive efforts to provide experimental facts in favour of **chromosomal theory of inheritance**. Bateson discovered linkage and Morgan's experiments have shown that the linkage could be broken by crossing over. Thus chromosomal maps of each chromosome was constructed through test cross data. The term gene was first given by Johannsen, 1909.

Muller (1925) defined mutations as an alteration of the gene. Muller 1940 proposed that a gene itself simultaneously is a unit of function, a unit of recombination and a unit of mutation. This concept of gene implied that gene may not possess any internal structure.

4.3 BIOCHEMICAL PHASE

Soon after the rediscovery of Mendel's work, **Garrod** 1909 proposed the hypothesis of Inborn errors of metabolism on the basis of certain metabolic diseases (alkaptonuria, albinism etc.).

Garrod's work involved relationship between genes and metabolic block. His concept of gene is



stated accurately as one mutant **gene–once** metabolic block, which about 40 years later was refined

Fig. 4.1 Seven characteristics in peas that were observed and scored by Mendel in his published experiments. As in other legumes such as various species of beans (*Phaseolus*), each flower bears a single ovary, containing upto 10 ovules, which then develops into the seed pod. Although "double fertilization"

occurs, the endosperm is soon resorbed in the pea, and the food reserves of the seed are contained in the two cotyledons (“Leaves”) of the embryo.

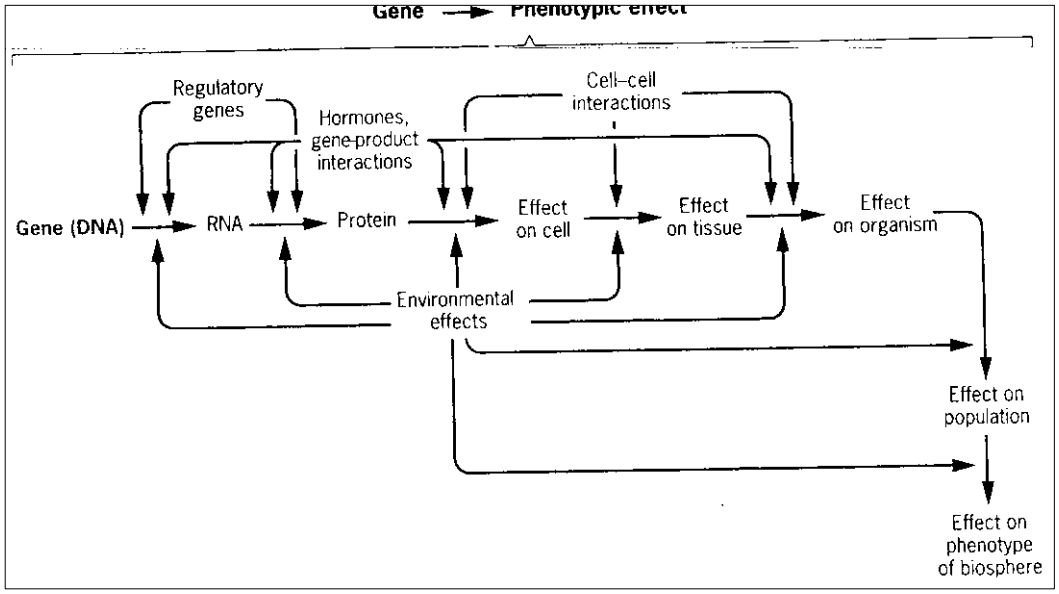


Fig. 4.2 The complex pathway by which a gene exerts its effect on the phenotype of an organism, a population, or the biosphere.

to the one gene – one enzyme caught enounciated by Beadle and Tatam, 1957. One of the inherited diseases, **alkaptonuria,** a blackening of urine upon exposure to air is due to the presence of homogenetisic acid in the urine, due to block in the normal pathway of metabolism of this compound. Thus Garrod clearly understood the relationship between genes and metabolism. His concept might be best stated as **one gene – one metabolic block**. However, during late 1930s, the investigation of eye pigments in *Drosophila* by Beadle and Ephrussi and the study of nutritional requirements in *Neurospora* by Beadle and Tatum (Fig. 4.3) made it clear that mutations cause the loss of enzyme activity indicating the enzyme catalyzed metabolic pathways are under genetic control. They proposed **one gene; and one enzyme hypothesis** and explained the action of each gene through the synthesis of a protein.

Thus upto 1930s, recombination was not believed to occur within genes and the gene was not considered subdivisible.

Thus, prior to 1940, genes were considered analogous to bead on a string, recombination occurred between, but not within genes. The gene was both the basic functional unit, which controlled one phenotype trait, and the elementary structural unit, which could not be subdivided by recombination or mutation. The results of pioneering studies by Oliver and Green were the first step towards the present concept of the gene, capable of mutating and recombining at different sites along its length and indicated that the gene was divisible.

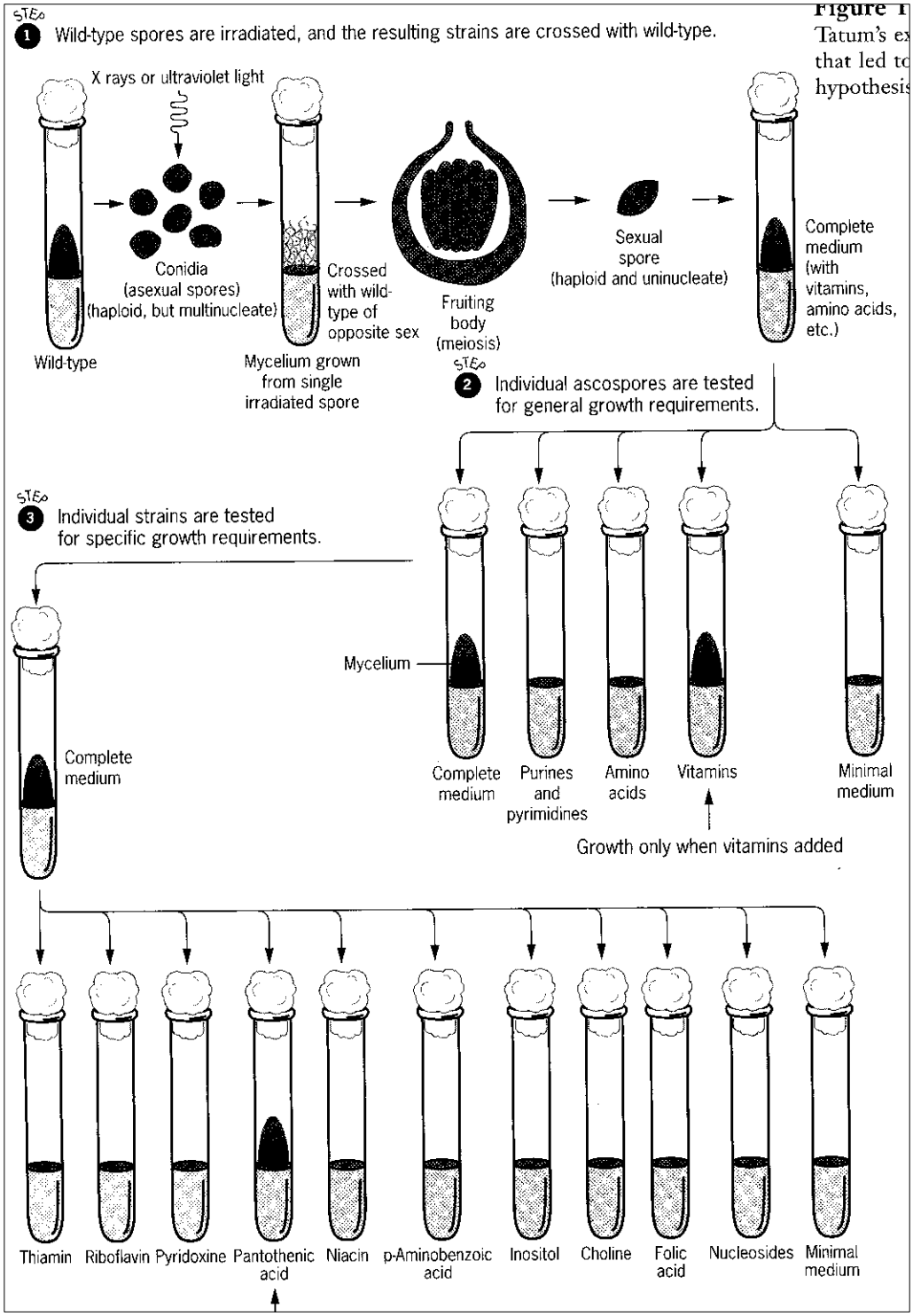


Fig. 4.3 Diagram of Beadle and Tatum's experiment with *Neurospora* that led to the one gene-one enzyme hypothesis.

In 1940, Oliver published first evidence indirectly that recombination could occur within a gene, while studying mutations at the lozenge locus on the X chromosomes of *Drosophila melanogasten* (Fig. 4.4). The identification of both products, the wild type and double mutant chromosomes produced by crossing over within lozenge gene, was noted by Green, one of Oliver's students.

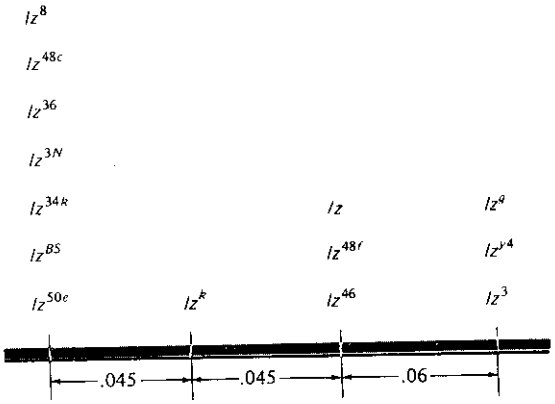


Fig. 4.4 Some alleles of *Iozenge* occupying different subloci within the *Iozenge* locus of *Drosophila melanogaster*.

4.3.1 One gene, One poplyptide

Subsequent to the work of Beadle and Tatum, many enzymes and structural proteins were shown to be heteromultimeric – they contain two or more different polypeptide chains, each polypeptide encoded by a separate gene e.g. hemoglobin, which transport oxygen from our lungs to all other tissues of our bodies, are tetrameric proteins that contain two α-globin chains and two β-globin chains. Other enzymes, *E. coli*, DNA polymerase III and RNA polymerase II (Lesson – DNA replication and gene expression) contain many different polypeptide subunits, each encoded by a separate gene. Thus **one gene – one enzyme concept** was modified to **one gene – one polypeptide**.

However, with the discovery of DNA structure by Watson and Crick, 1953, the chemical nature of gene in terms of nucleotide sequences was proposed. Subsequently, it was revealed that nucleotide sequences determined the amino acid sequence of polypeptides.

Benzer extended the picture of gene by demonstrating the existence of 199 distinct sites of mutation, separable by recombination within rIIA gene of bacteriophage T_4. Benzer's located the existence of many mutational sites within rII locus and implied that the locus possessed an intricate fine structure (see chapter - Genetic, Recombination in Phage). Benzer's picture of gene as a sequence of nucleotide pairs, capable of mutating at many distinct sites was soon verified by the results of many researchers, investigating gene structure in several different organisms, both prokaryotes and eukaryotes.

Given this information about the structure of genes, it followed that the smallest unit of genetic material capable of mutation might be a smaller part of the gene, a single nucleotide pair and was termed muton, recombination might occur between adjacent nucleotide pairs whether between or within genes. Thus unit of recombination (recon) was also found to be much smaller. Thus on the basis of complementation test, **cistron** was defined, while **muton** and **recon** were defined by the recombination.

4.4 MOLECULAR PHASE

Since 1970s, Recombinant DNA technology was used to characterise gene structure and organisation in prokaryotes as well as eukaryotes. In 1977, Glover and Hogness and Chamber discovered that animal viruses and eukaryotes have **split genes**. The structure of mammalian beta-globin gene is an example of split gene (Fig. 4.5). Thus, genes possess coding sequences called **exons** and non-coding sequences **introns**. According to Gilbert introns might perform two functions – they may enhance recombination of lengthening a gene, they may allow one gene to make several polypeptides (see lesson Gene Expression).

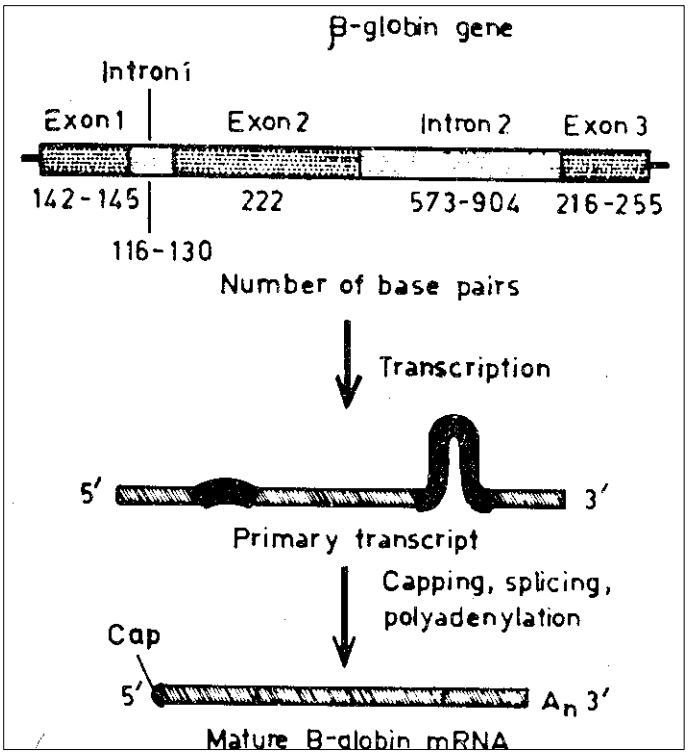


Fig. 4.5 Structure of the mammalian beta-globin gene, an example of split gene. Note the presence of introns and exons and how the introns are removed during the maturation of the mRNA. Figures denote the number of amino acids.

An analysis of eukaryotic genomic DNA by a variety of molecular techniques has revealed that eukaryotic genome differs from prokaryotic genome in having large amounts of non-coding and repetitive DNA (See Lesson I, Unit-III, Physical and Chemical Structure of DNA).

Orgel and Crick (1980) have argued that a large part of non-coding DNA has no function other than to ensure its own survival (Selfish DNA).

The non-coding DNA includes introns, regulators, pseudogenes (does not produce a functional product), Satellite DNA and Selfish DNA. The introns account for upto 90% of a gene. The highly repetitive DNA accounts for about 90% of the genome. The extent of such DNA is highly variable among different organisms. The discovery of transposable elements that jump not only within the genome but from one species to other are significant revolutions in the area of genetics (See transposons lesson). The findings on pseudogenes and transposable elements has challenged our concept of gene structure, stability and mutation (See lesson transposons).

4.4.1 Complex gene

Most prokaryotic genes consist of continuous sequences of nucleotide pairs, which specify sequence of amino acids in the polypeptide gene products. Most eukaryotic gene are split into expressed sequences (**exons**) and intervening sequences (**introns**). Nevertheless, transcripts of **split genes** may undergo several different types of splicing, making the relationships between genes and proteins more complex than one gene – one polypeptide relationship.

The alternate splicing pathways (See lesson Gene Regulation) are tissue specific, producing related proteins that carry out, not necessarily identical functions in different types of cells, e.g. where alternate splicing pathways give rise to two or more different polypeptides, each antibody contains two light chains and two heavy chains (Fig. 4.6), a variable region which exhibits extensive diversity (Fig. 4.7) from antibody to antibody and a constant region which is largely the same in all antibodies.

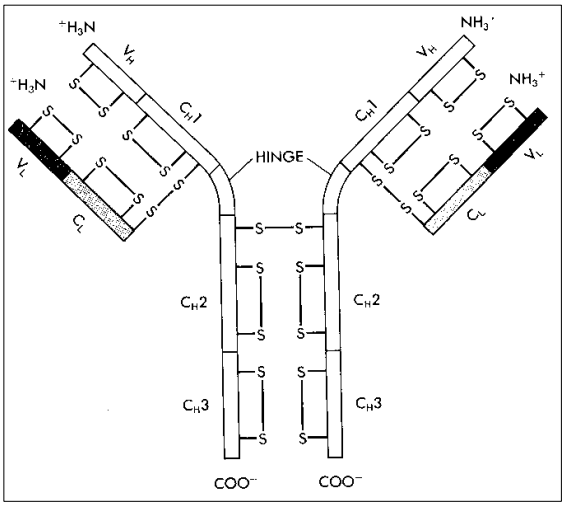


Fig. 4.6 The structure of an antibody protein. Two light chains (color) and two heavy chains (white) are held together by disulfide bonds. The light chains and the heavy chains each contain one variable unit (V_L or V_H) at their amino-terminal ends. The light chains also contain one constant unit (C_L); the heavy-chain constant portion has four domans (C_H1, C_H2, C_H3, and the hinge region).

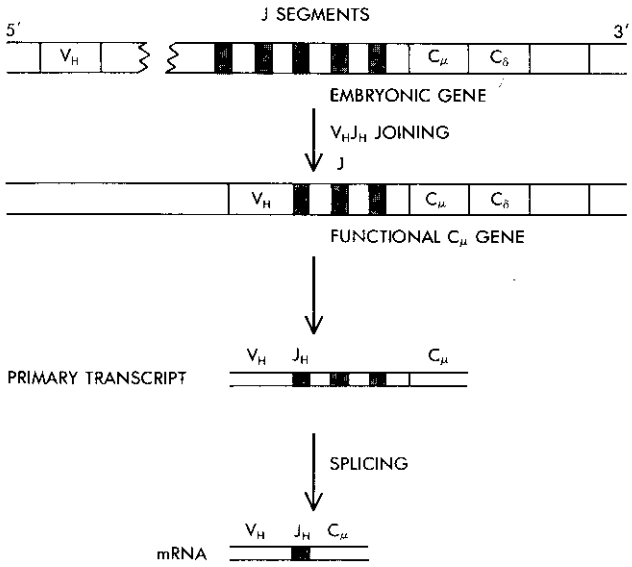
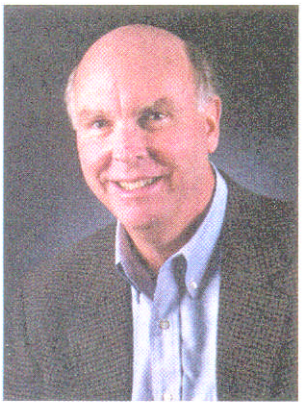


Fig. 4.7 A V_H gene is linked to a C_H gene by means of a J (joining) segment that is located in a cluster of such segments upstream of the C_H genes. After the initial recombination event, RNA splicing removes all of the other J segmenets to produce the mature mRNA.

4.4.2 Genomics revolution

The development of DNA sequencing techniques by Maxam and Gilbert (1977) and Sanger (1977), enabled scientists to read DNA or gene sequences representing 4 nitrogen bases (Fig. 4.8). Sanger *et al*., 1977, were the first to sequence the entire genome of a virus ϕ x 174, consisting of 5375 bases and identified 7 genes.

Francis Collins



J. Craig Venter

Given the one gene – one polypeptide relationship, geneticists have asked how many genes it takes to produce a fly, a worm, a plant or a human. The answer to this gene numbers question has been a major surprise to **human genome sequencing** teams 2001 (Celera, Inc and International Human genome sequencing consortium). Human genome – How many genes? The answer is only 30,000 to 40,000 genes.

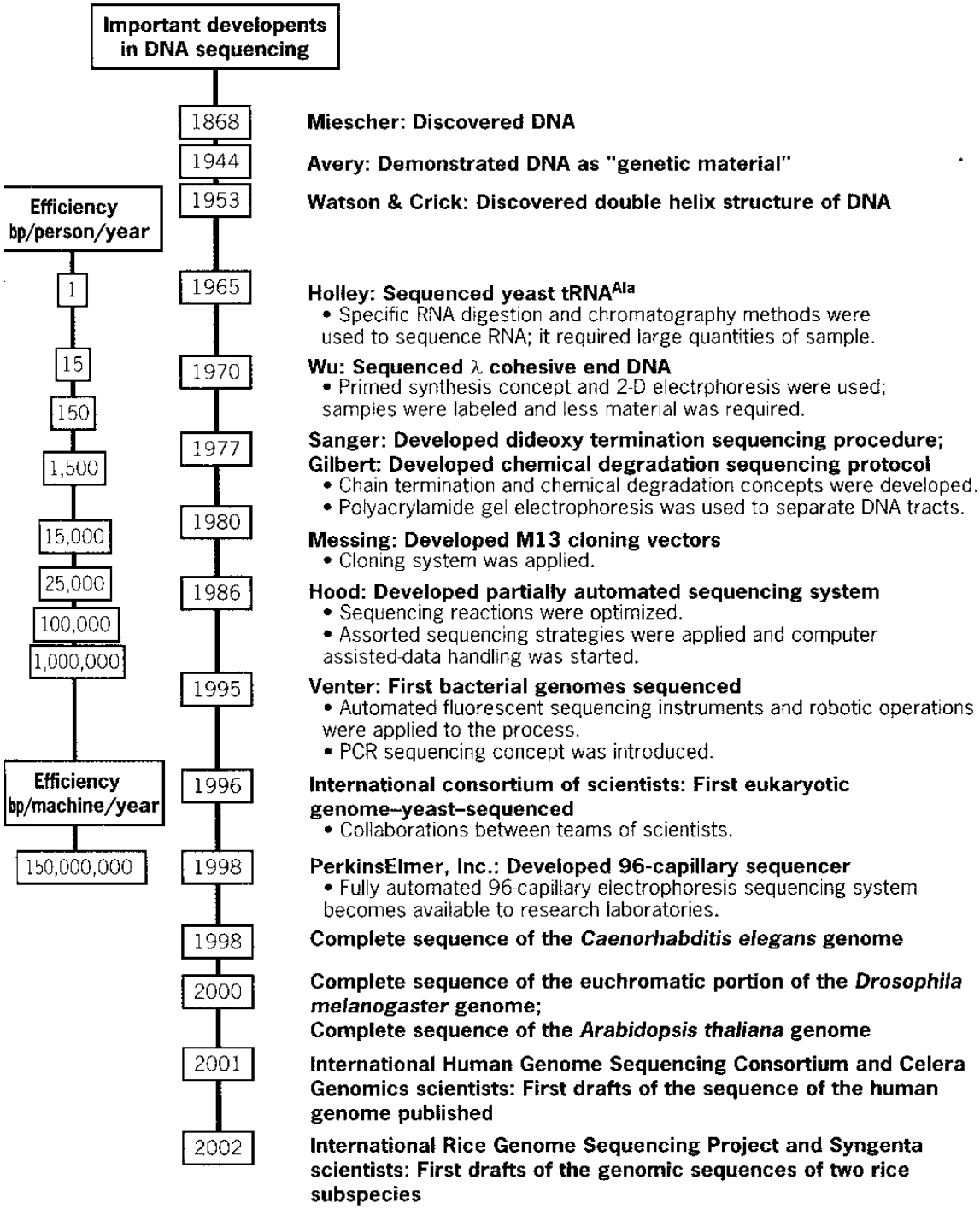


Fig. 4.8 Advances in DNA sequencing efficiency, some of the technological developments that enhanced the productivity of sequencers, and some landmarks in DNA sequencing. Initially, all the steps in DNA sequencing were performed manually, making it a very labor-intensive process. However, fully automated sequencing machines have now largely replaced human sequencers, greatly increasing efficiency.

The world's fastest gene hunter Craig Venter entered the genomics by setting up his own institute named TIGR in USA (The Institute of Genomic Research) in 1992).

The year 2000 marks a century since the rediscovery of Mendels laws. The entire biological scientific community has been working to reveal the secrecy of genes and witnessed the whole genome sequence information of 600 viruses and viroids, 200 plasmids, 185 organells, 50 eubacteriae, 11 archeea, 1 fungus, 4 animals and 2 plants (*Arabidopsis* and rice) etc.

Looking back from today, we have travelled a long distance from rediscovery of Mendel's factors in 1900. The year 2000 was marked by the sequencing of entire genome of *Drosophila* with 14,000 genes (Adams *et al*., 2000). *Arabidopsis thaliana*, the first plant genome sequences with 26,000 genes (AGI Consortium, 2000) and one of the world's food crop rice (*Oryza sativa* 450 Mbp genome sequenced (Bloom, 2000).

The understanding of gene structure and function as reviewed above has brought revolution in genomics culminating into a phase – Bioinformatics, which is the management of and analysis of biological information stored in databases. This multidisciplinary scientific tool developed, involving various disciplines of science like biology, mathematics, computer science and information technology etc.

4.5 SUMMARY

The concept of gene from Mendel's work to 21st century is reviewed under different phases. The rediscovery of Mendel's factors, one gene one metabolic block by Garrod, one gene one polyptide relationship by Beadle and Tatun are described. Benzer's work on r II locus of phage revealed the gene as subdivisible unit of cistron, a functional unit, recon, a recombinational unit, muton a mutational unit. Split gene concept and gene splicing pathways and genomic revolution is briefly described.

4.6 MODEL QUESTIONS

1. Describe briefly the Evolution of gene concept.
2. Write notes on:
   a) One gene – One polypeptide concept
   b) Split gene
   c) Antibody diversity

4.7 REFERENCE BOOKS

i) **Essential Genetics** by P.J. Russell, Blackwell Scientific Publication, 1987.
ii) **Modern Genetics** by F.J. Ayala & J.A. Kiger Jr. The Benjamin/Cummings Publ. Com. 1980.

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Prof. T.N. MARY

M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-IV

Lesson 1

Physical and Chemical Structure of DNA

3.1 INTRODUCTION

From the earlier lesson, the important properties of genetic material and DNA as the genetic material is understood from the experiments of Griffith 1928; Avery Macleod and McCarty 1944 and Hershey and Chase 1952. The genetic information of all living organisms, except RNA viruses is stored in DNA. The nature of the chemical subunits of DNA and RNA, the double helical structure of DNA, different form of DNA are described in this lesson.

1.2 CHEMISTRY OF NUCLEIC ACIDS – DNA AND RNA

Nucleic acids are macromolecules composed of nucleotides. Each nucleotide is composed of: 1) a phosphate group, 2) sugar, and 3) a nitrogen base (either one ring or two rings). In DNA, the sugar is deoxyribose; in RNA sugar is ribose (Fig. 1.1). Four bases are found in DNA: adenine, guanine, thymine and cytosine. RNA contains all these bases but has uacil in place of thymine. Adenine and guanine are double-ring bases called purines; cytosine, thymine and uracil are single-ring bases called pyrimidines. (1.2) These subunits are joined together in long chains. The structural components of nucleic acids and deoxyribonucleotoides present in DNA are shown (Fig. 1.3).

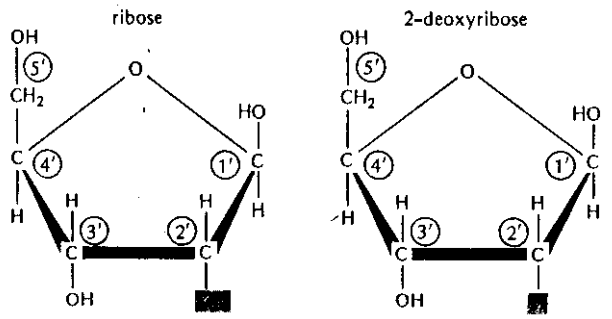


Fig. 1.1 Structural formulas for ribose and deoxyribose sugars. Carbon positions on these pentose sugars are designated by primed numbers 1' to 5'.

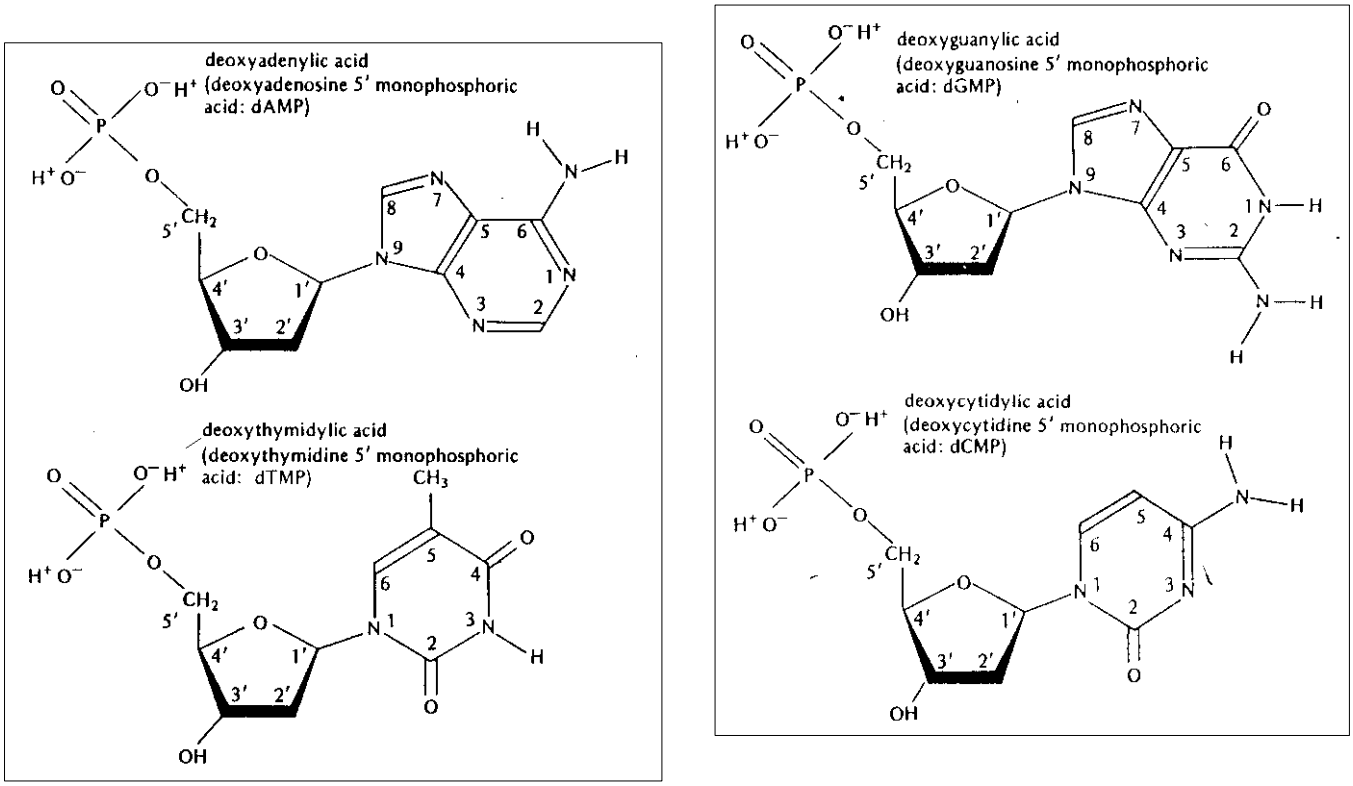
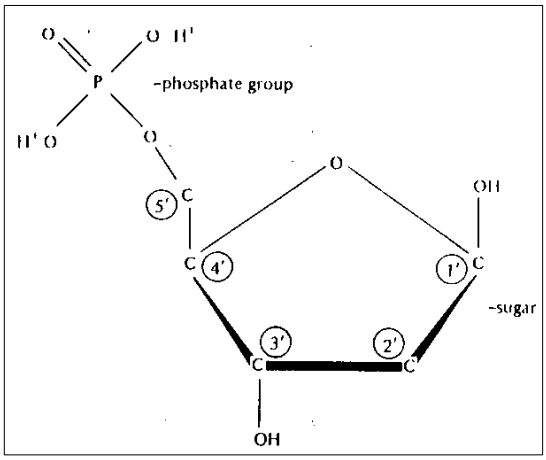
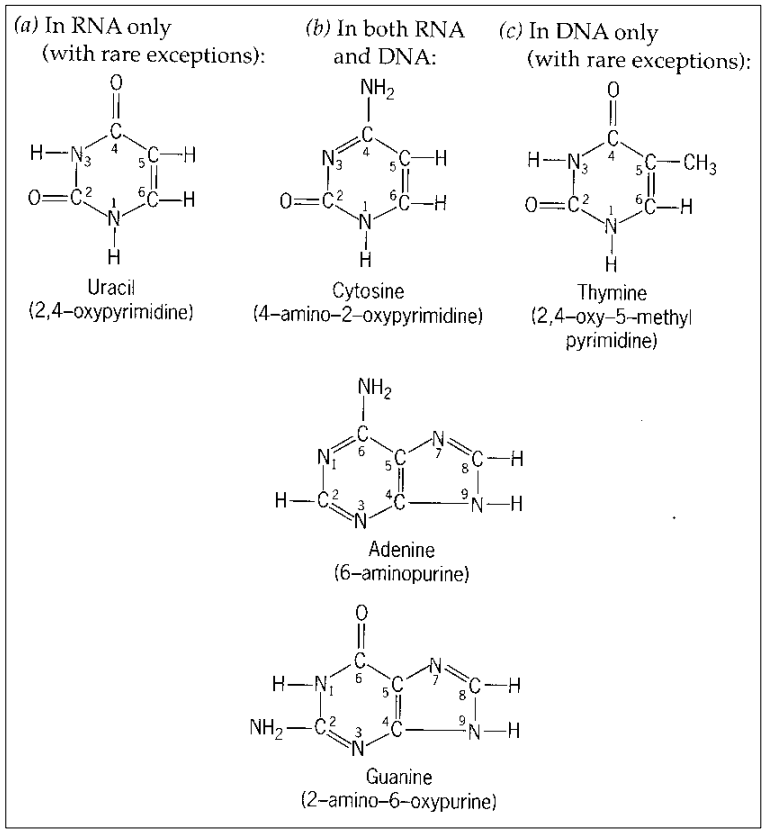


Fig. 1.2 Structural formulas of the common bases in nucleic acids. Numbering of the ring positions follows the Chemical Abstracts system.

A nucleotide is formed by attachment of base to the first carbon of the sugar and attachment of a phosphate to the 5' carbon of the same sugar. (Fig. 1.2a) Nucleotides of DNA are linked together by the formation of a bond between the phosphate of one nucleotide of 5' carbon atom and hydroxyl group (OH group) at 3' carbon of adjacent molecule. Long strings of nucleotides can be polymerized by phosphodiester bonding.



(a)



(b)

Fig. 1.3 The four major nucleotides in DNA. Carbon atoms on the sugar are designated by prime superscripts, and positions on the ring structures of bases are designated by plain numbers.

The sugar contains five carbons and is called ribose. When ribose lacks one oxygen atom on 2' carbon, it is called deoxyribose when phosphate group is attached it is phosphorylated sugar (Fig. 1.2a). Although these are the sugars in nucleic acids, any particular nucleic acid does not contain both of these sugars at the same time. As a consequence, there are two kinds of nucleic acids, ribonucleic acid (RNA) found commonly in the cytoplasm and deoxyribonucleic acid (DNA) found with few exceptions only in the nucleus.

Bases containing one carbon-nitrogen ring are the pyrimidines (Fig. 1.2b), two ring bases are the purines. In DNA, the two pyrimidines are cytosine and thymine, while RNA carries both cytosine and uracil. The difference between thymine and uracil is due to the presence of methyl group CH_3, instead of a hydrogen H at 5 position of thymine. Thymine is replaced by uracil in RNA.

In addition to the difference in ring structures, an amino group (NH_2) is present at 4^{th} position of one pyrimidine (cytosine) and 6^{th} position of purine (adenine) while keto group (C=0) occurs at the position (6^{th}) in other bases (thymine, aracil and guanine) Fig, 1.2b). Thus, DNA and

RNA can also be described as containing two kinds of amino bases and two kinds of ketobases evenly divided between pyrimidines and purines.

One of the exciting breakthroughs in the history of biology of occurred in 1953, when Watson and crick deduced the correct structure of DNA. Watson and Crick's double helical structure was based on three lines of evidences. Chemical nature of components of DNA, X-ray crystallography and Chargaff's ratio.

1.3 X-RAY CRYSTALLOGRAPHY

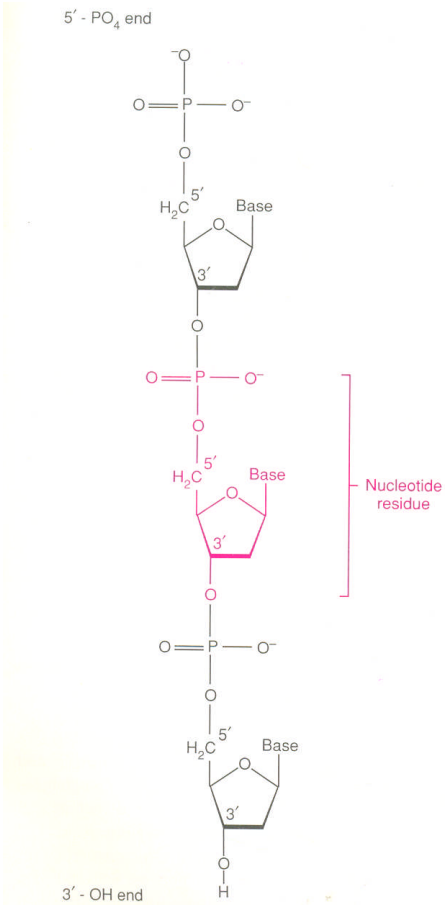
When X-rays are focused through molecules, they are deflected by the atoms of the molecules in specific patterns, called diffraction patterns. These X-ray diffraction patterns can be recorded on x-ray film. The nature of this pattern depends on the structure of the crystal (Fig. 3). Mauria Wilkins, Rosalind Franklin and their colleagues were using X-ray crystallography (in the same laboratory of Kings College, London) where Watson and Crick to analyse the structure of DNA. The cross in the centre of the photograp indicates that the molecule is a helix, the dark areas at top and bottom come from the bases, stacked perpendicularly to the main axis of the molecule (Fig. 1.5).

1.4 CHARGAFF'S RATIO

Chargaff analyzed the composition of DNA from different organism, and found the concentration of Adenine was equal to that of thymine and the concentration of cytosine was always equal to the concentration of guanine. Their data showed that the total concentration of pyrimidines was always equal to purine. Thus $\frac{\text{adenine + thymine}}{\text{cytosine + guanine}}$ ratio varied widely in DNAs of different species (Table 1).

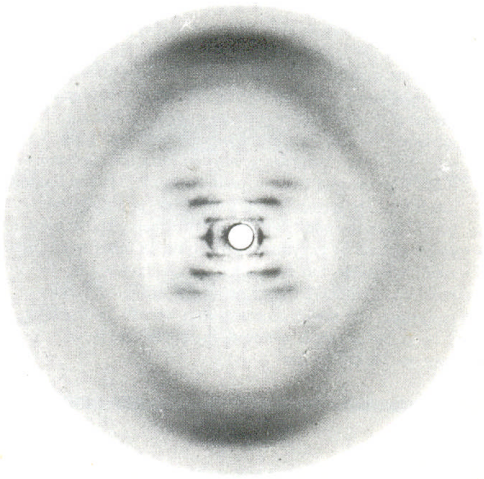
Table 1. Percentage Base Composition of Some DNAs

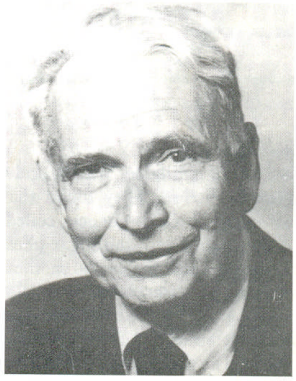
Species	Adenine	Thymine	Guanine	Cytosine
Human Being (Liver)	30.3	30.3	19.5	19.9
Mycobacterium tuberculosis	15.1	14.6	34.9	35.4
Sea Urchin	32.8	32.1	17.7	18.4



Rosalind E. Franklin (1920–1958)
Courtesy of Cold Spring Harbor Laboratory.

Fig. 1.4 Fig. 1.4 Polymerization of adjacent nucleotides to form a sugar-phosphate strand. There is no limit to the length that the strand can be, or the type of base attached to each nucleotide residue.





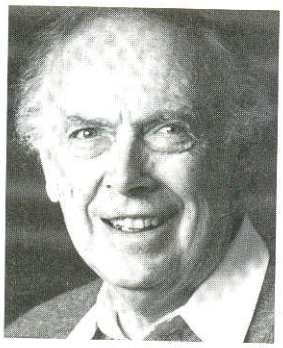
Erwin Chargaff (1905–)
Courtesy of Dr. Erwin Chargaff.

Fig. 1.5 Scatter pattern of a beam of X rays passed through crystalline DNA.

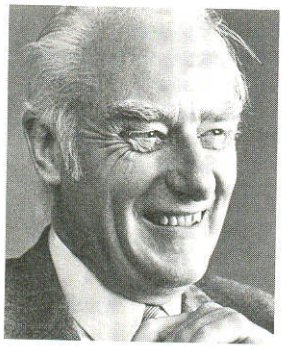
1.5 DNA STRUCTURE

Watson-Crick Model of DNA structure

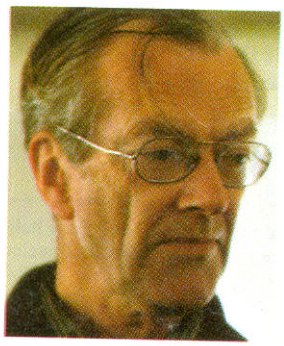
On the basis of above Chargaff's data, Wilkins and Franklin's X-ray diffraction data, Watson and crick proposed that DNA exists as a right handed double helix, with two



James D. Watson (1928–)
Cold Spring Harbor Laboratory Research Library Archives. Margot Bennet, photographer.



Francis Crick (1916–)
Reproduced by permission of Herb Weitman, Washington University, St. Louis, Missouri.



Maurice H. F. Wilkins (1916–)
Courtesy of Dr. Maurice H. F. Wilkins and Biophysics Department, King's College, London.

polynucleotide chains coiled about one another in a spiral manner. Two strands are said to be complementary and held together by hydrogen bonds giving stability to the molecule, two hydrogen bonbs between A=T, three between C=G. The base pairing is specific, adenine always pairs with thymine, guanine always pairs with cytosine.

The base pairs in DNA are arranged about 3.4 Å apart, with 10 base pairs per turn of the double helix. The base pairs are hydrophobic (insoluble in water). This hydrophobic nature contributes considerable stability to DNA molecule present in the aqueous protoplasm of living cells. The sugar-phosphate backbones of the strands are antiparallel (Fig. 1.6). The phospodiester bonds in one strand move from 3′ carbon of one nucleotide to 5′ carbon of adjacent nucleotide, whereas in complementary strand move from 5′ carbon to 3′ carbon. Thus they both are antiparallel. This opposite polarity of the complementary strands of DNA double helix plays important role in DNA replication, transcription and recombination.

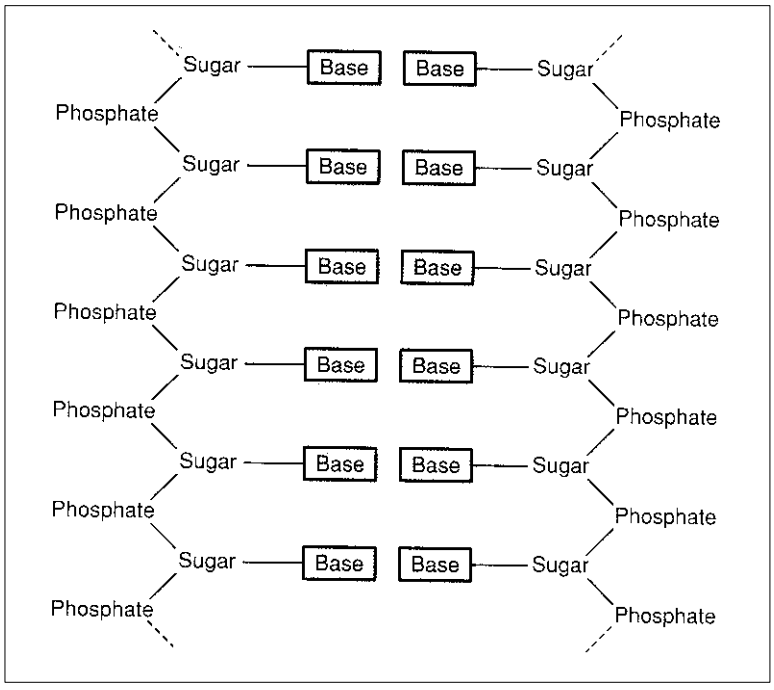


Fig. 1.6 Double helical structure of DNA. (a) Component parts.

Double helix is a stable structure

Several factors account for the stability of the double helical structure of DNA.

i. Both internal and external hydrogen bonds stabilize the double helix. In DNA, H-bonds form between A=T and C≡G bases.

ii. The negatively charged phosphate groups are all situated on the exterior surface of the helix in such a way that they have minimal effect on one another and are free to interact electrostatically with cations in solution, such as Mg^{++}.

iii. The core of the helix, which consists of base pairs, is bonded together with hydrophobic interactions and van der Walls forces besides H bonding.

Taking into account, the facts known at that time, Watson and Crick proposed a double helix structure of DNA as shown in Fig. 1.6. According to them,

1) DNA comprises two polynucleotide chains.

2) The nitrogen bases are stacked on the inside of the helix, while the sugar-phosphate forming the backbone of the molecule is on the outside.

3) The two polynucleotide chains interact by hydrogen bonding. There is a very strict combining rule for hydrogen bonding.

4) Ten base pairs occur per turn of the helix. The pitch of the helix is 34Å, meaning that the spacing between the adjacent base pairs is 3.4 Å . The helix diameter is 20 Å.

5) The two strands of the double helix are antiparallel, with one polynucleotide chain running in $5' \rightarrow 3'$ direction and the other chain in $3' \rightarrow 5'$ direction. Only antiparallel polynucleotides form a stable double helix.

6) The double helix has two different grooves: major and minor. Thus, the helix is not absolutely regular. This feature is important in the interaction between the double helix and proteins involved in DNA replication and expression.

7) The double helix is right handed.

8) The two polynucleotide chain base pairs are complementary. The sequence of one determines the sequence of the other. This complementary base pairing is of fundamental importance in molecular biology.

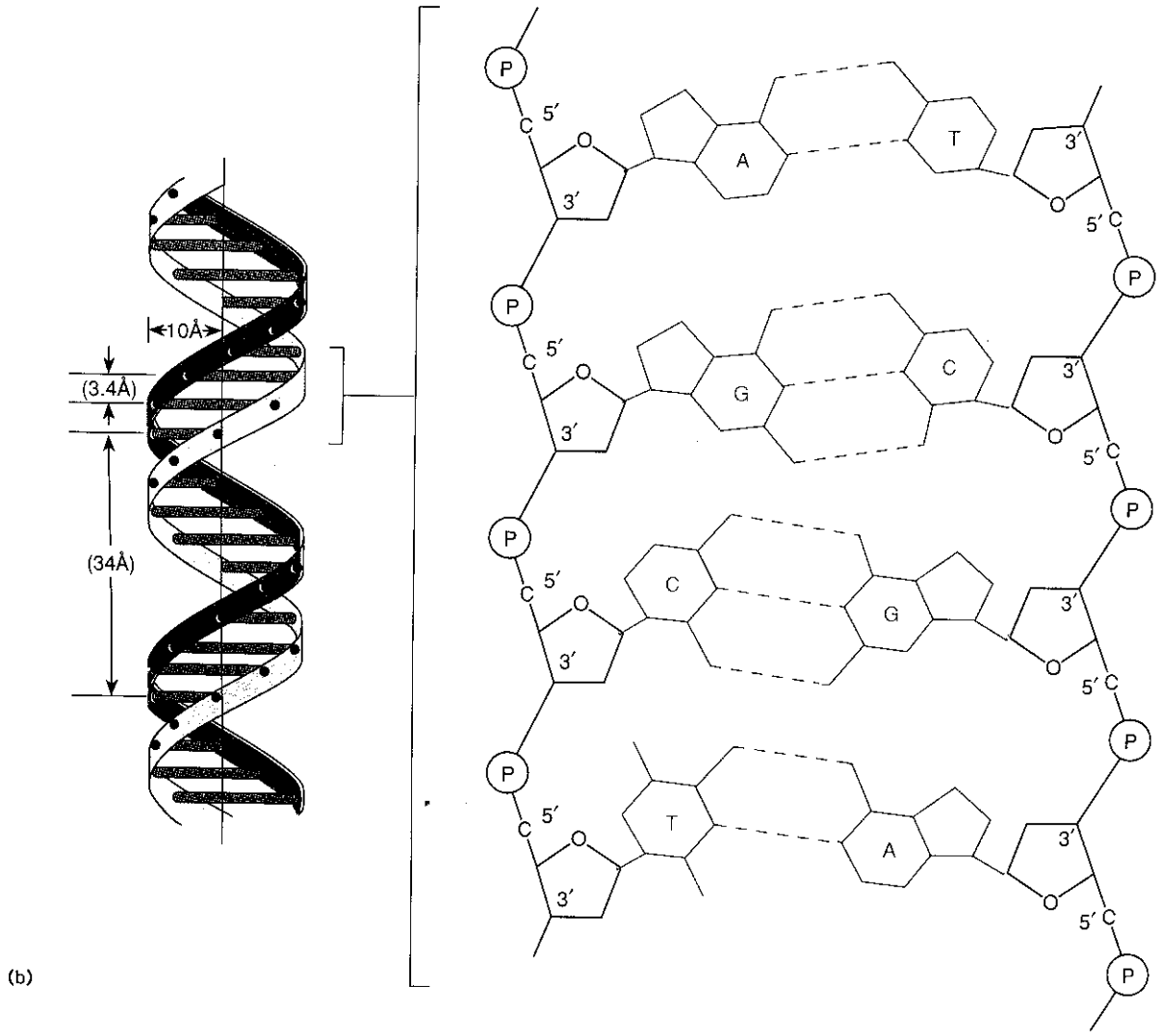


Fig. 1.6 Double helical structure of DNA. (b) Line drawing.

1.6 ALTERNATE FORMS OF DNA

DNA in aqueous solutions of low concentrations of salts remain as B DNA. Majority of DNA molecules in aqueous protoplasm of living cells exist in B conformation only. Watson and Crick Model is of B DNA. The DNA molecule can exhibit considerable flexibility depending upon the cell environment. In high concentration of salts, DNA exist as A-DNA with 11 nucleotide pairs per turn. Certain DNA sequences have been shown to exist in a left-handed, double helical form called Z-DNA. It differs from A & B conformations in having 12 base pairs per turn (Table 2). lexander Rich and his colleagues discovered a left handed helix that they called Z DNA (Fig. 1.7).

DNA molecules are super coiled. This super coiling causes DNA to collapse as telephone cord (Fig. 1.8). The DNA molecules of all organisms from viruses to large eukaryotes, exhibit negative super coiling. Negative supercoiling is involved in replication, recombination, gene expression.

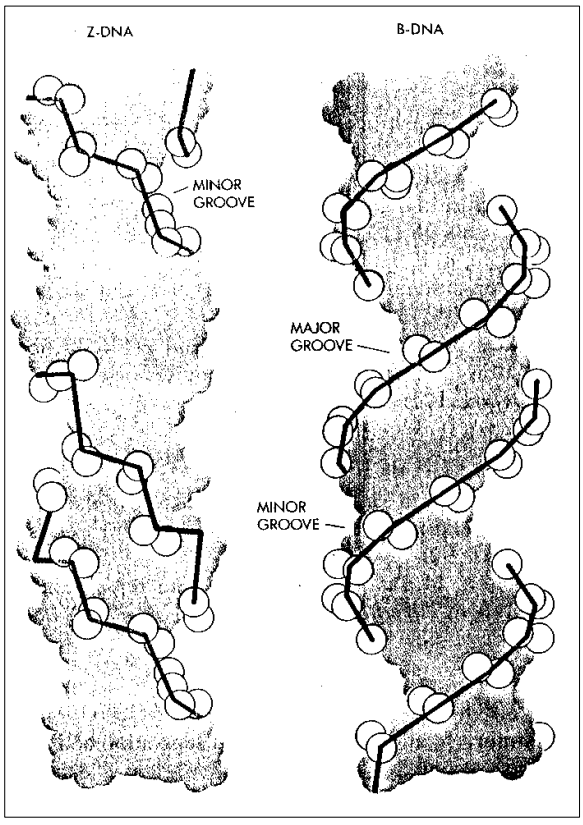


Fig. 1.7 Left-handed (Z-) DNA forms a zigzag helix

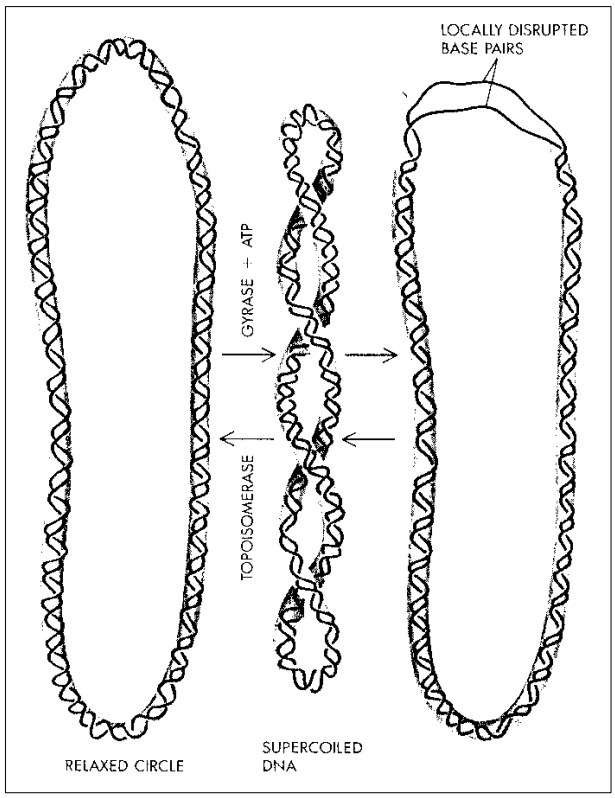


Fig. 1.8 A relaxed circular DNA molecule can be twisted into a negatively supercoiled molecule by the action of DNA gyrase. The reverse reaction is catalyzed by topoisomerase ("nicking-closing" enzyme). The strain in the negatively supercoiled form can be relieved by local disruption of the double helix to produce single-stranded regions

Genome complexity: A lare portion of DNA in Eukaryotic cells does not code for protein. Much of this DNA consists of multiple repeats. The coding regions of genes are interrupted by infron sequences. Much of the non-coding DNA consisting of multiple repeats.

Unique sequence: In *E. coli* genome, all the DNA has a unique sequence since it consists of single copy genes.

Moderately repetitive: Much of the moderately repetitive DNA comprises a number of DNA sequences of a few hundred base pairs, each repeated more than 100,000 times and scattered through out the genome e.g. rRNA genes, histone genes 5-80 per cent of genome of *Arabidopsis* and transposable elements.

Highly repetitive: Much of this repetitive DNA is present in telomeres and centromeres. They exist as 10^5 copies per genome. In *Drosophila virilis*, three sequences each seven base pairs long 5'-AT AAACT-3', 5'-ACAAACT-3' and 5'-ACAATT-3' form distinct satellite bands (Fig. 1.9). They don't encode proteins. What there are the function of these highly repetitive DNA sequences? At present there is no clear answer except that is carried along by the replication and junk DNA process of segregation of chromosome.

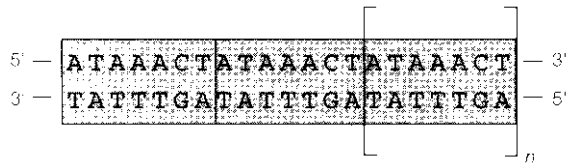


Fig. 1.9 *Drosophilla satellite* DNA repeat

1.7 SUMMARY

Following the lines of evidence showing by Wilkins, Franklin X-ray Crystallography Chargaffs ratio, Watson and Crick, 1953 discovered the double helical structure of DNA is described. The chemistry of subunits of DNA i.e. Nucleotides, purines, pyrimidines etc., was outlined. Alternate forms of DNA and their differences were brought out genome complexity is explained while comparing *E. coli* and Eukaryotic genome.

1.8 MODEL QUESTIONS

1. Describe the double helical structure of DNA.
2. Write about different types of DNA you have studied.
3. What are the evidences that lead to the discovery of structure of DNA by Watson and Creek.

4. Write notes on:

   a) Difference between DNA-RNA.
   b) Polynucleotide
   c) Antiparallel nature of DNA strands
   d) Phosphodiester bond
   e) Differences in organization of DNA between prokaryotes and eukaryotes.

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M.Sc. BOTANY (Final)

Paper-VII CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-IV

Lesson 2

Replication of DNA

2.1 INTRODUCTION

The human life emerges from a single cell and that cell gives rise to hundreds of billions of cells during fetal development since DNA replicates during each cell division. Cellular machinery responsible for DNA replication must work more importantly. Indeed the fidelity of DNA replication is amazing. In this chapter, the emphasis is on key features of the mechanism by which accurate replication of DNA occurs. The synthesis of DNA occurs in S phase of interphase, in the cell cycle before Mitosis. It involves three steps: (1) chain initiation, (2) chain elongation, and (3) chain termination.

During 1953 when structure of DNA was published by Watson and Crick, they suggested that the replication of the double helix could take place by unwinding of the DNA, so that each strand would form a new double helix by acting as a template for a newly synthesized strand (Fig. 2.1).

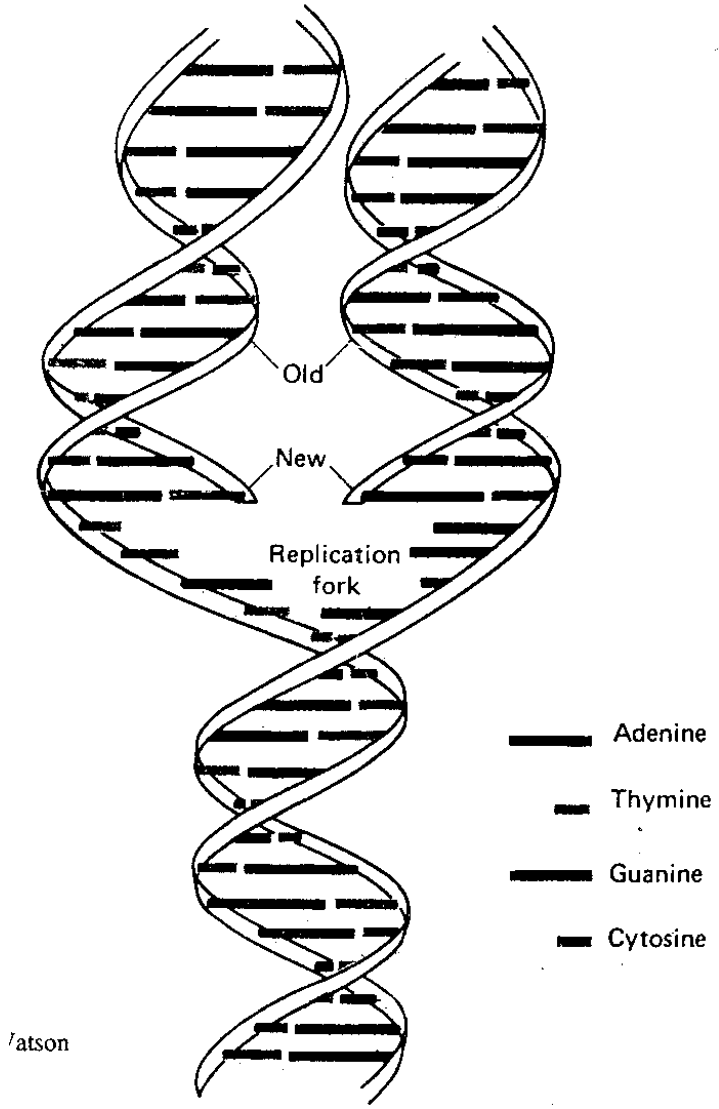

Fig. 2.1 The replication of DNA according to Watson and Crick

Hypothetically DNA replication can occur by any three different mechanisms. 1) Semi-conservative mechanism, where new strand is synthesized against template strand. Conservative mechanism in which two strands are conserved and directing the synthesis of a new progeny double helix. (3) A dispersive mechanism, with segments of parental and progeny strands interspersed due to synthesis and rejoining of short segments of DNA (Fig. 2.1A).

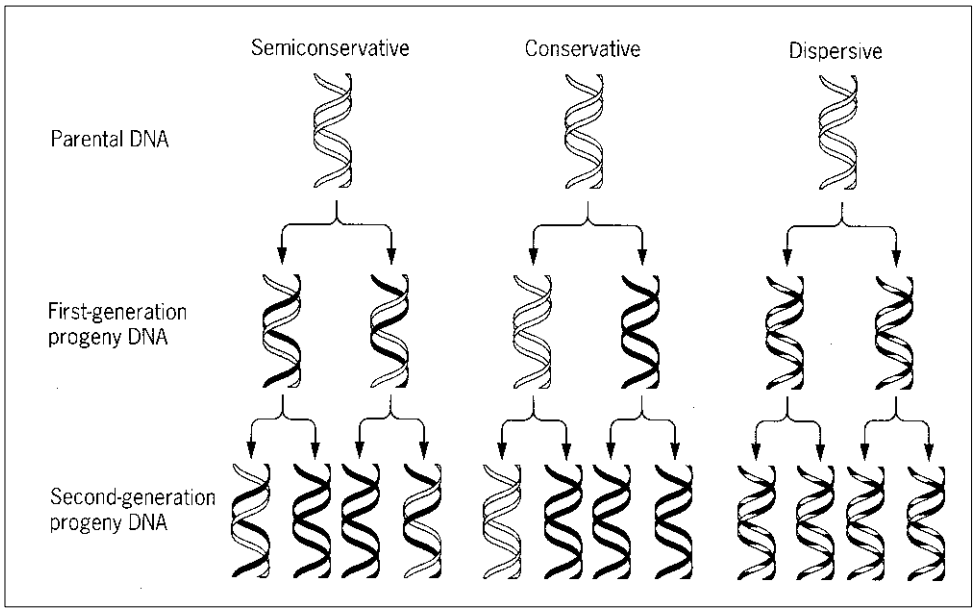

Fig. 2.1A Three possible modes of DNA replication: (1) semiconservative, in which each strand of the parental double helix is conserved and directs the synthesis of a new complementary progeny strand; (2) conservative, in which the parental double helix is conserved and directs the synthesis of a new progeny double helix; and (3) dispersive, in which segments of each parental strand are conserved and direct the synthesis of new complementary strand segments that are subsequently joined to produce new progeny strands.

2.2 EXPERIMENTS CONFIRMING DNA REPLICATION

2.2.1 Meselson and Stahl in *E. coli*

The experiments demonstrating the semi-conservative mechanism of DNA replication was performed by Meselson and Stahl (1958) in common colon bacillus *Escherichia coli*, a prokaryote; by a procedure called equilibrium density-gradient centrifugation using cesium chloride. Because the density of most DNAs is about the same as the density of heavy salts such as cesium chloride (CsCl).

The density of 6M CsCl is 1.7 g/cm^3
E. coli DNA containing ^{14}N has 1.710 g/cm^3
E. coli DNA containing ^{15}N has 1.724 g/cm^3

Therefore, it is easy to separate DNA based on the density layers formed according to density gradient centrifugation method.

Meselson and Stahl grew *E. coli* cells for many generations in the presence of heavy isotope of nitrogen, ^{15}N and transferred them to medium containing ^{14}N. After the cells were allowed to

grow in the presence of ^{14}N for varying periods of time, the DNAs were extracted and analyzed in CSCl equilibrium density gradients. The results were consistent only with semi-conservative replication.

All the DNA isolated from cells after one generation of growth in medium containing ^{14}N had a density halfway between heavy DNA (^{15}N) and light DNA (^{14}N). This is referred as hybrid density. After two generations of growth in 14N medium, it is 1:1 ratio of hybrid : light DNA. After three generations 1:3 ratio of hybrid : light DNA (Fig. 2.2).

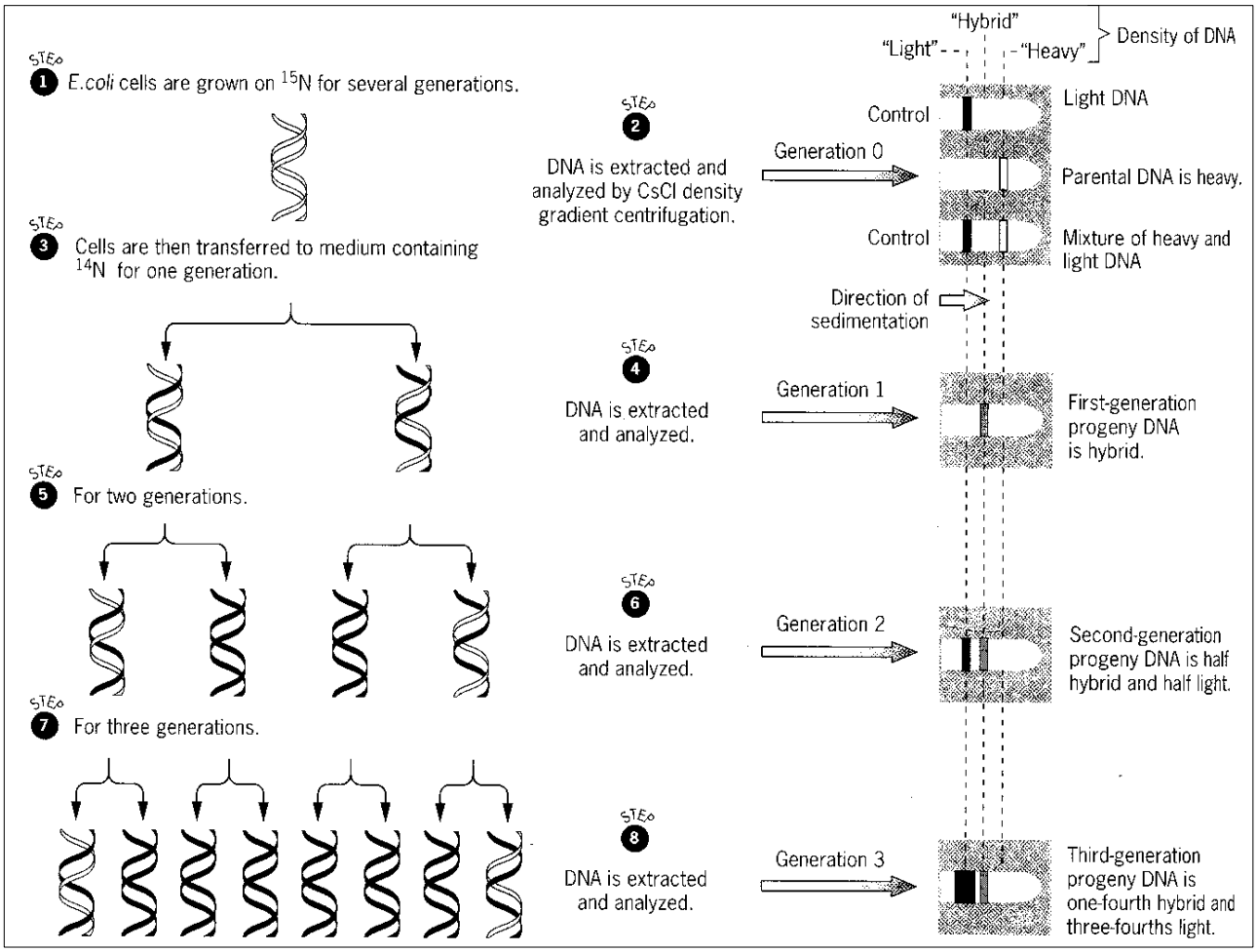

Fig. 2.2 Meselson and Stahl's experiment demonstrating that DNA replicates by a semiconservative mechanism in *E. coli*. The diagram shows that the results of their experiment are those expected if the *E. coli* chromosome replicates semiconservatively. Different results would have been obtained if DNA replication in *E. coli* were either conservative or dispersive.

Conservative replication would not produce any DNA molecules with hybrid density. If replication were dispersive Meselson and Stahl would have observed a shift of DNA from heavy

towards light in each generation. Since the observed results of Meselson and Stahl are proving the semi-conservative replication of DNA, it is accepted hypothesis, that DNA replication takes place by semi-conservative mechanism.

2.2.2 Taylor and his colleagues in Broad Bean

The semi-conservative replication of eukaryotic chromosome was demonstrated in 1957 by the results of experiments carried out by J. Herbert Taylor, Philip Woods and Walter Hughes in root tip cells of broad bean, *Vicia faba*. Taylor and colleagues labed *Vicia faba* chromosomes by growing root tips for eight hrs in medium containing radioactive ^{3}H-thymidine. The root tips were removed from radioactive medium and transferred to non-radioactive medium containing alkaloid colchicine. Thus the number of chromosomes per nucleus will double once per cell cycle in the presence of colchicine. This doubling of chromosome number each cell generation allowed Taylor and his colleagues to determine how many DNA duplications each cell had undergone subsequent to the incorporation of radioactive thymidine (Fig. 2.3). The gross structure of replicating bacterial chromosome was first determined by John Cairns (1963) by autoradiography (Fig. 2.4).

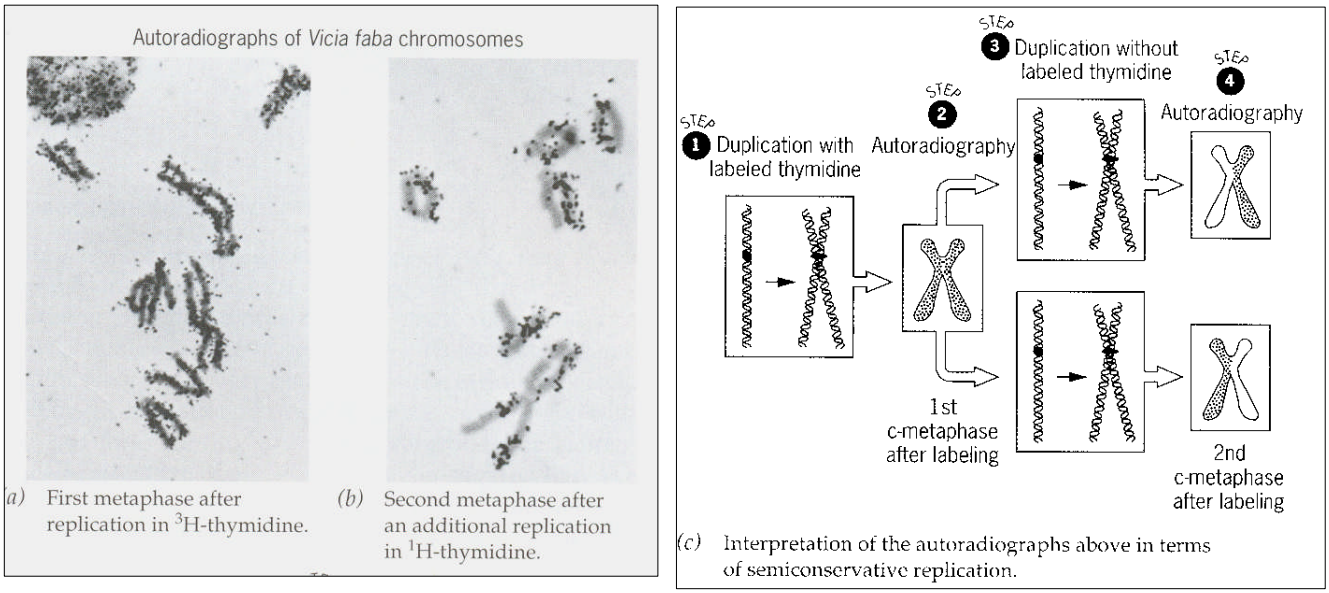

Fig. 2.3 Results and interpretation of the experiment by taylor and colleagues showing that the chromosomal DNA of the broad bean, Vicia faba, replicates by a semiconservative mechanism.

Fig. 2.4 Visualization of the replication of the E. coli chromosome by autoradiography. One of Cairns' autoradiographs of a θ shaped replicating chromosome from a cell that had been grown for two generations in the presence of ^{3}H-thymidine, with his interpretative diagram shown at the upper left.

2.3 MECHANISM OF DNA REPLICATION

2.3.1 Replicons

In prokaryotes, the replication starts at a specific site, called origin or replicon and proceeds bidirectionally. Each Y-shaped structure is a replication fork and two replication forks move in opposite directions around the circular chromosome as a result, an intermediate theta structure is formed (Fig. 2.5).

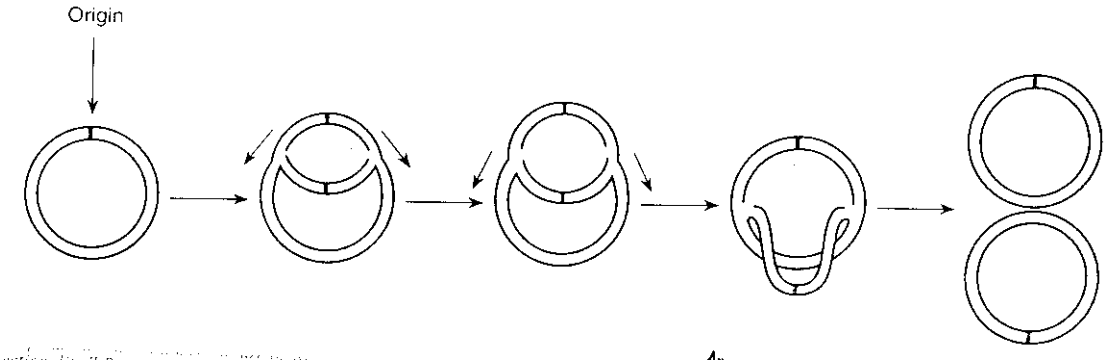

Fig. 2.5 Observable stages in the DNA replication of a circular chromosome, assuming bidirectional DNA synthesis. The intermediate figures are called theta structures

In eukaryotes since the DNA molecules (chromosomes) are larger than in prokaryotes and are not circular, there are multiple sites of initiation of replication. Thus eukaryotic chromosome is composed of many replicating units (Fig. 2.6). The DNA of Drosophila in largest chromosome contain 6.5×10^7 nucleotide pairs. The rate of replication is 2600 nucleotides per minute at 25°C. Thus single replication fork take 17.5 days to replicate one of these giant DNA molecules. Since DNA in eukaryotic interphase chromosome is packed at 10 nm nucleosomes, nucleosome disassembles into two half-nucleosomes during replication and reassemble after replication (Fig. 2.6A).

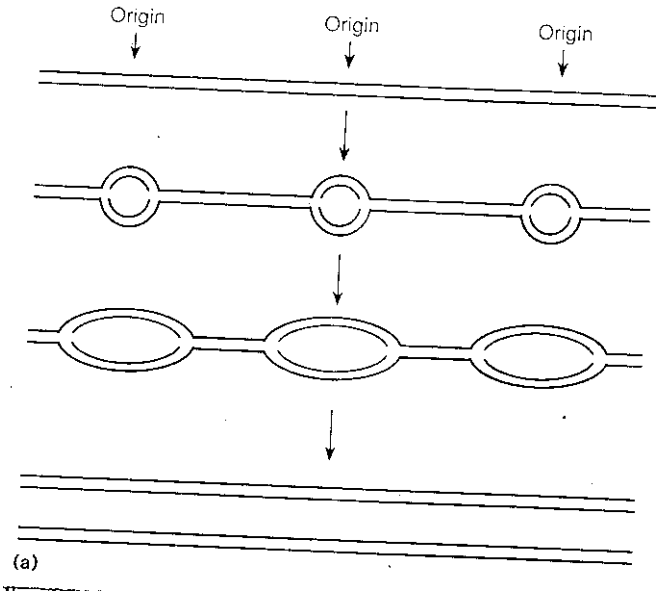

Fig. 2.6 Replication bubbles. Formation of bubbles (eyes) in eukaryotic DNA because of multiple sites of origin of DNA synthesis

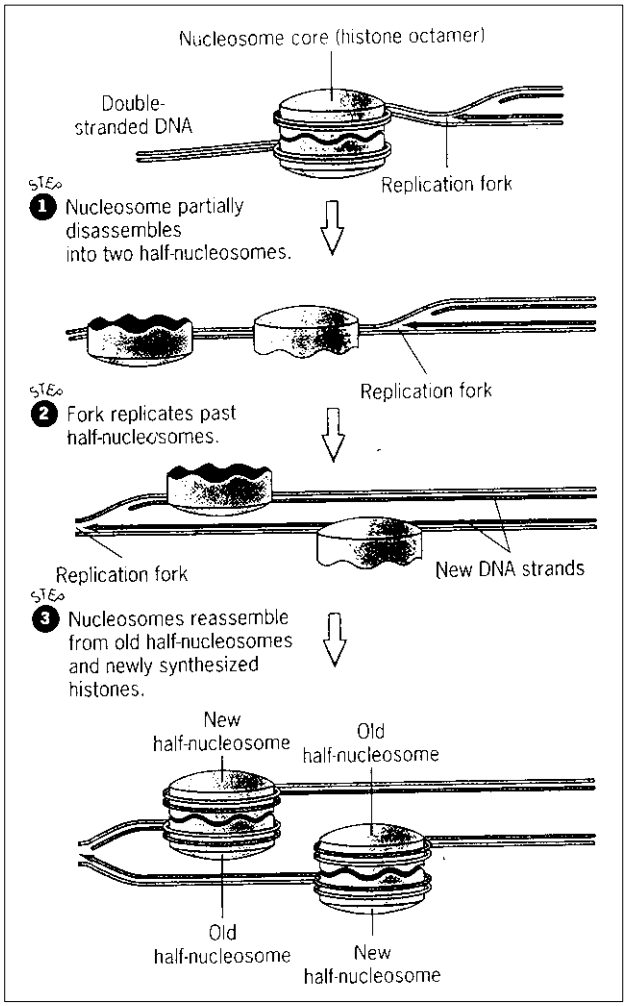

Fig. 2.6A Movement of a replication fork past nucleosomes. (a) Electron micrograph showing nucleosomes on both sides of two replication forks in *Drosophila*. Recall that DNA replication is bidirectional; thus, each branch point is a replication fork, (b) One model for the movement of replication forks past nucleosomes.

2.3.2 Enzymology of DNA replication

DNA polymerase-I initially called as korenberg's enzyme. It is a repair enzyme. It removes mismatched base pairs.

DNA polymerase-II – It does small portion of 5' → 3' polymerase and 3' → 5' exonuclease activity
DNA polymerase-III – It does 5' → 3' polymerase and 3' → 5' exonucleane activity.

Polymerase-III is the true replicate enzyme (Fig. 2.7) for semi conservative replication of DNA in *E. coli*, is a complex enzyme composed of many different subunits. Recently polymerase IV and V are also identified involved in replication of damaged DNA.

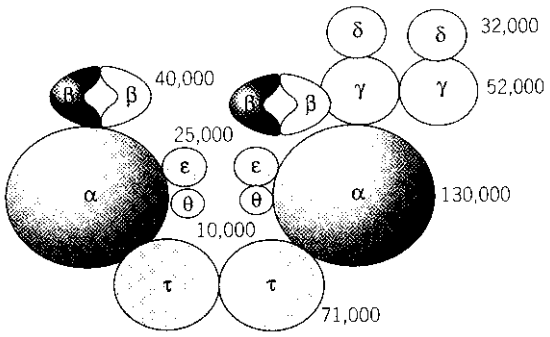

Fig. 2.7. Structure of the E. coli DNA polymerse III holoenzyme. The numbers give mass in daltons.

Eukaryotic organisms are more complex with seven different DNA polymerases. They are named as α, β, γ, δ, ε, ς and n and their activity is given in the Table 1.

Table 1. Activity of Polymerases

Prokaryotes	**Eukaryotes**
DNA polymerase I DNA repair	α, ς and ε - replication of Nuclear DNA
DNA polymerase-II polymerase activity	X-replication of DNA in mitochondria
DNA polymerase III major enzyme activity in replication	β, ς , α, *n* – DNA repair enzymes
DNA polymerase IV & V replication of damaged DNA	

2.3.3 Initiation of DNA chains

The unwinding of DNA is catalyzed by DNA helicase (Single strand building Fig. 2.10). They are maintained in unmound state by SSB proteins. Each new DNA chain is initiated by a short RNA primer synthesized by DNA primase, deoxyribonucleotides are added to the RNA primer by DNA polymerase. Since both strands are synthesized against a template strand, it is bidirectional replication but each strand synthesis occurs in 5' → 3' direction only. Primers provide free 3'-OHs required for extension of polynucleotide chains by DNA polymerase.

2.3.4. Continuous and Discontinuous DNA Replication (Semi discontinuous)

One strand, the leading strand synthesis is made continuously in 5' → 3' direction from the origin. The second strand is lagging strand initiated at the replication fork proceeds back towards origin by forming Okazaki fragments (Fig. 2.8). They are of 1000-2000 bases in length in prokaryotes short but 100-200 bales only in eukaryotes.

DNA polymerase I completes synthesis by removing RNA primer, replaces it with DNA nucleotides. These are joined by an enzyme DNA ligase.

As the replication fork moves along a parental double helex, two DNA strands are replicated in a coordinated steps (Fig. 2.9). The complete replication apparatus moving along the DNA molecule at a replication fork is called the replisome. It contains DNA polymerase III, holoenzyme, one core replicates the leading strand, the second core replicates lagging strand. Thus primosome unwinds DNA molecule and synthesize RNA primers. A single replisome with two copies of DNA polymerase, a helicase, a primosome moves along the DNA to complete replication (Fig. 2.10).

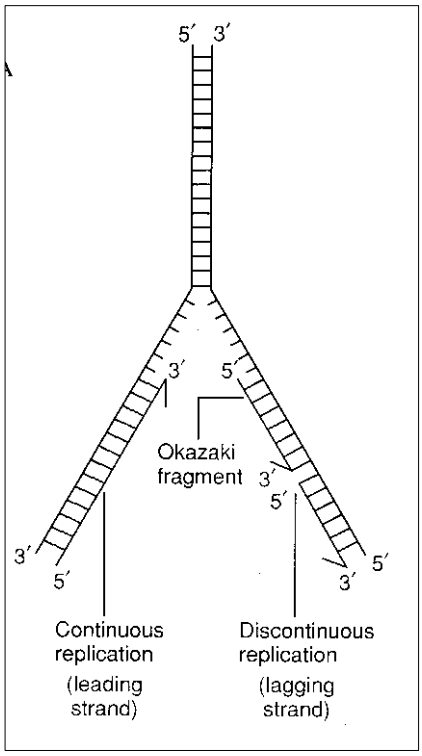

Fig. 2.8 Discontinuous model of DNA replication

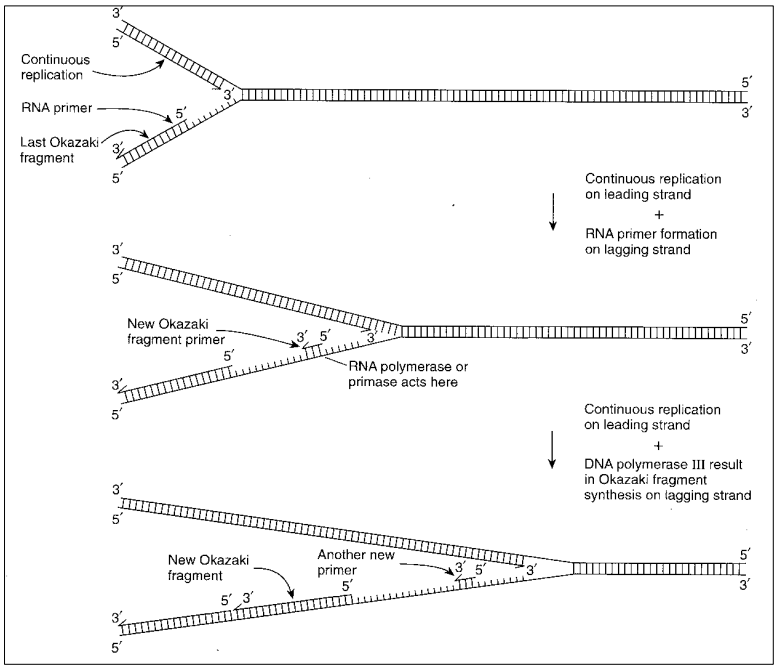

Fig. 2.9 Primer formation and elongation create an Okazaki fragment during discontinuous DNA replication

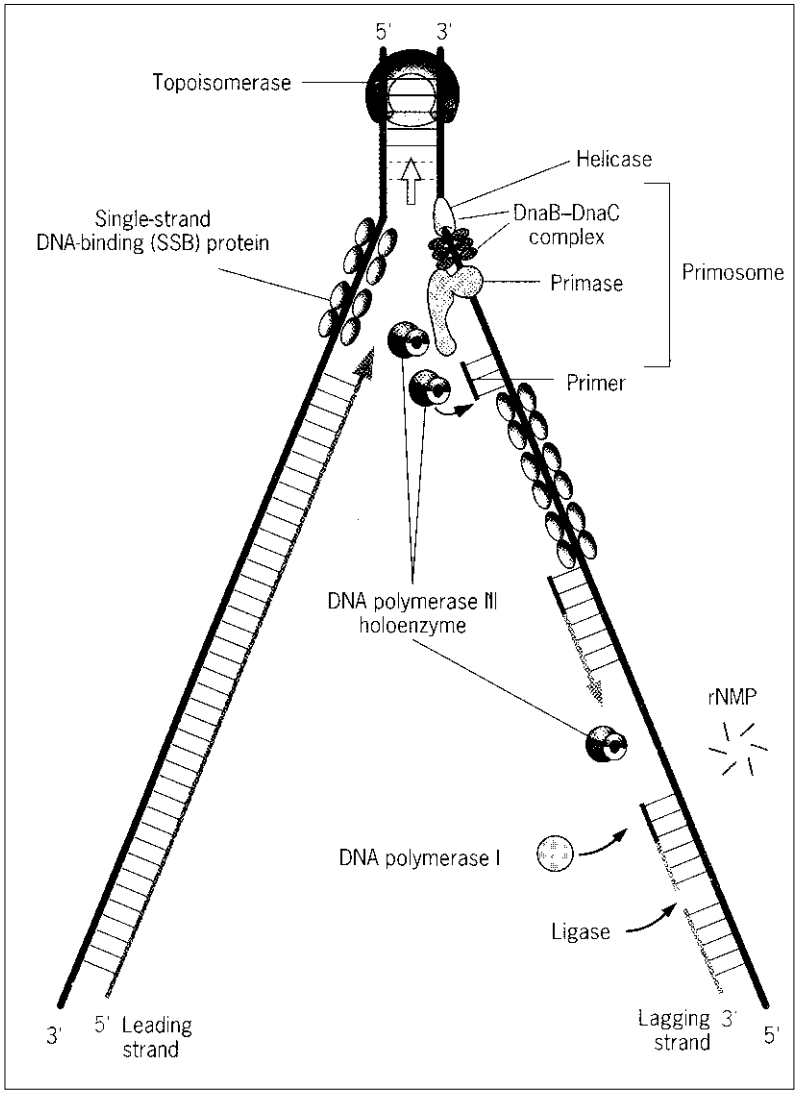

Fig. 2.10 Diagram of a replication fork in *E. coli* showing the major components of the replication apparatus. rNMP = ribonucleoside monophosphates.

2.4 OTHER MODELS OF DNA REPLICATION

2.4.1 Rolling-circle Model: This form of replication occurs in the F plasmid, during conjugation. In this model, the nick creates a free 3'OH end. Synthesis of a new circular strand occurs by addition of nucleotides to the 3' end using complementary strand as a template. As nucleotides are added to the broken strand in a continuous fashion, the other end is displaced as 5'-PO_4 tail. When the tail is replicated in discontinuous manner, the linear DNA is rolled into a closed circle; by DNA ligase which closes the replicated circular strand (Fig. 2.11).

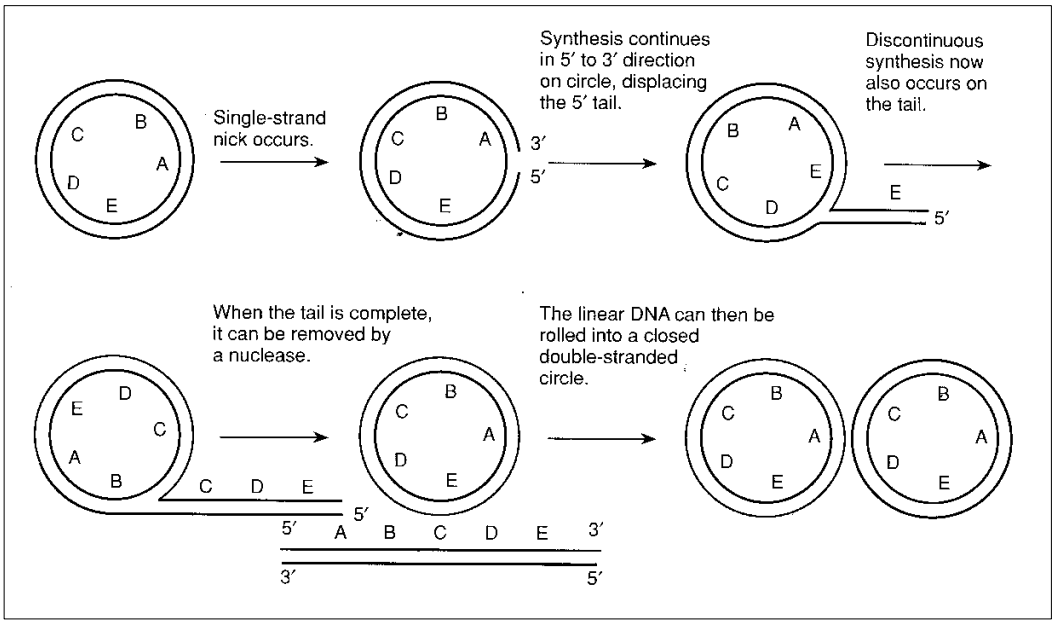

Fig. 2.11 Rolling-circle of DNA replication. The letters A-E provide landmarks on the chromosome

2.4.2 D-Loop Model: Chloroplasts and mitochondria have their own circular DNA molecules. Replication begins on one strand, displacing the other, forming a displacement loop or D-loop structure. Replication continues until the process passes the origin of replication on the other strand: thus resulting two circles of DNA molecules (Fig. 2.12).

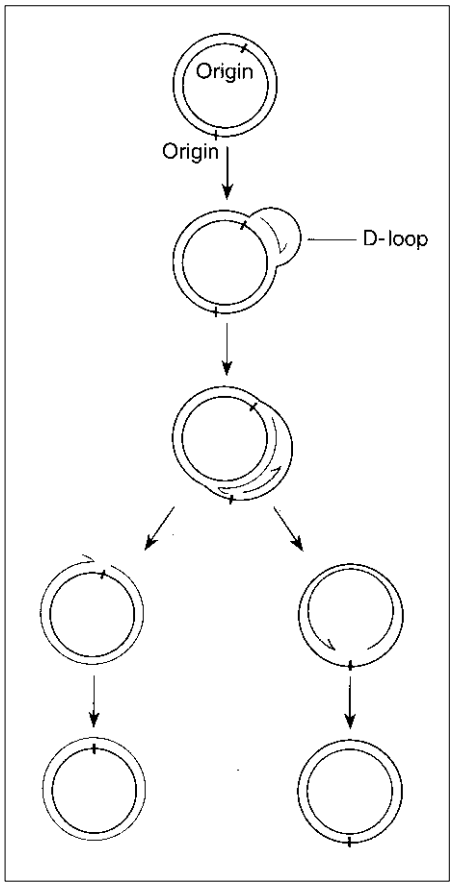

Fig. 2.12 D-loops form during mitochondrial and chloroplast DNA replication because the origin of replication is at different places on the two strands of the double helix →

2.5 SUMMARY

Semi conservative model of DNA replication was confirmed through Meselson and Stahls cesium chloride equilibrium density gradient centrifugation using isotopes of nitrogen. Prokaryotic chromosomes are circular with a single replicon. Eukaryotic DNA is linear with multiple replicons. DNA synthesis is continuous on one strand, a leading strand synthesis and discontinuous on the other strand, lagging strand synthesis by formation of Okhaza fragments. Though it is

bidirectional, but strand synthesis is always 5 $\rightarrow$ 3 direction only. The function of enzymes involved and the comparative account of the mechanism between prokaryotes and eukaryotes are discussed.

2.6 MODEL QUESTIONS

1. Describe the experiments of Meselson and Stahl involved in DNA replication.
2. Discuss in detail the mechanism of DNA replication.
3. In what ways does chromosomal DNA replication in eukaryotes differ from DNA replication in prokaryotes.
4. Write short notes on the following:
   a) Replicon.
   b) Rolling circle model
   c) DNA polymerases

2.7 REFERENCE BOOKS

1. **Instant Notes Molecular Biology** by Turner, P.C., McLennan A.D. Bates & MRH White, Viva Books Ltd., 2001.
2. **Principles of Genetics** by Tamarin, 2000.
3. **Principles of Genetics** by Peter Snustad and M.J. Simmons, 2003.
4. **Recombinant DNA** by Watson, Gilman, Wittkowsky and Zoller, 1992.
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7. **Genetics** by P.K. Gupta, 1996.
8. **Advanced Molecular Biology** by Wymann, 1998.
9. **Genetics** by Frefeilder.

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Prof. T. N. MARY

M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-IV

Lesson 3

DNA repair mechanisms

3.0 **Objective:** The mechanisms of DNA repair, their importance in human health is well recognised. These DNA repair mechanisms have evolved, to safeguard the integrity of genetic information in living organisms which is discussed below:

3.1 INTRODUCTION

Radiations, chemical mutagens, aberrant climatic conditions, spontaneous decay are constantly damaging DNA. It is estimated that there are several thousand bases in a cell. Given the key role that DNA plays in growth and development of living organism, the evolution of mechanisms to protect its integrity would seem inevitable. What is more important to the survival of a species than maintaining the integrity of its genetic blue print?

In general, skin cells contain enzymes that correct the changes in DNA, resulting from exposure to ultraviolet light. However, people suffer from inherited skin disease **xeroderma pigmentosum** due to the failure, to repair DNA damage. It is an autosomal recessive trait. Individuals with this disease, develop skin tumors after exposure to sunlight.

Therefore, in the long evolutionary challenges, cells have evolved many mechanisms to repair the damaged or incorrectly replicated DNA. Without the repair of DNA in an organism, phenotypic characters, functions would have changed at a rapid rate in an uncontrolled fashion. Thus to prevent effects of mutation, cells have acquired DNA repair mechanisms, which are

important to the survival of a species and they can be studied under five categories as described below in *E. coli* cells.

3.2 DNA REPAIR MECHANISMS

3.2.1 Light-dependent repair

It is otherwise called as photoreactivation of DNA in bacteria, carried out by light-activated enzyme called photolyase. When DNA is exposed to u.v. light, **thymine dimers** are produced in DNA. They use light energy to clear the covalent cross links. **Photolyase** is produced in the presence of light in *E. coli* cells, which cleaves the bonds between thymine bases, from the energy derived from visible light (Fig. 3.1). This enzyme thus reverses the u.v. induced dimerization.

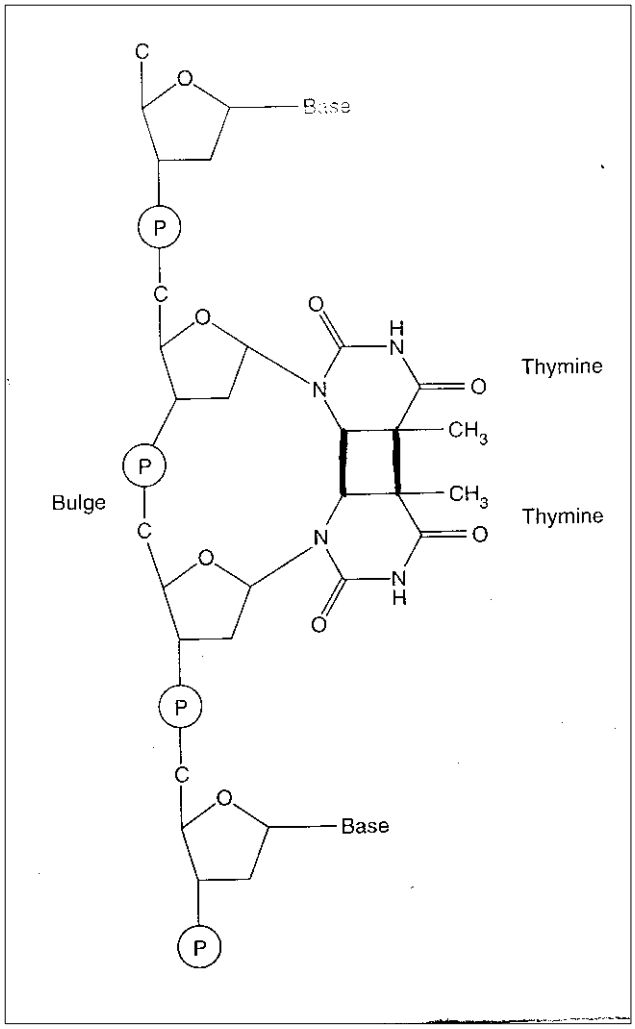

Fig. 3.1 UV-induced dimerization of adjacent thymines in DNA.
The broad lines represent the dimer bonds iin theadjacent thymines.

3.2.2 Excision Repair (Dark Repair)

This mechanism is not dependent on light. It involves three steps. DNA repair endonuclease enzyme recognizes, binds to and excises the damaged bases in DNA. In step two, a DNA polymerase-I fills in gap by using undamaged complementary strand of DNA as template. Excision repair systems are found in all organisms, from largest viruses to eukaryotes. Thus, it is a simple system of locating, excising and repairing the damage caused by u.v. light. In step 3, the enzyme DNA ligase seals the break, left by DNA polymerase to complete the repair process (Fig. 3.2).

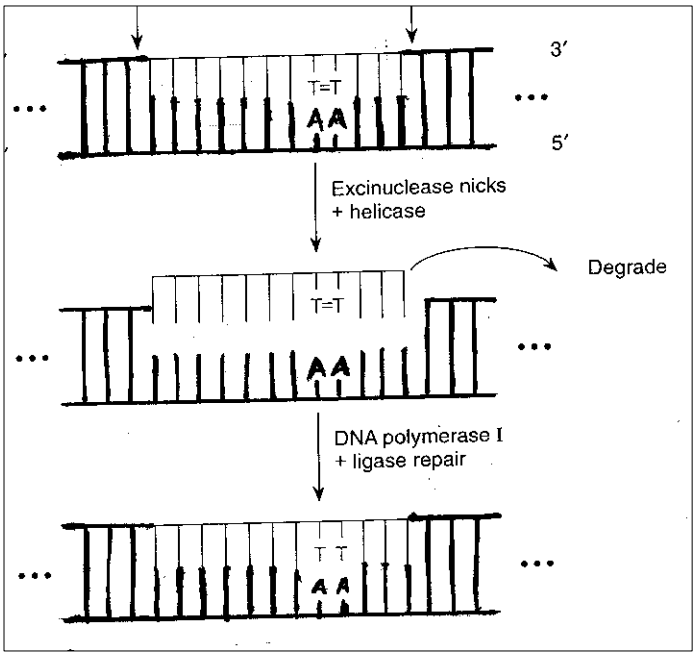

Fig. 3.2 Mechanism of excision repair of thymine dimers. A 12-bp segment of DNA with the dimer is removed by an exonuclease and a helicase. Repair by DNA polymerase I and DNA ligase patch the duplex.

3.2.3 Mismatch Repair

It is responsible for 99% of all repairs of DNA. As DNA polymerase replicates DNA, some errors are not corrected by the proof reading capability of the polymerase e.g. G can be paired with C rather than T. Such errors are recognized by mis match repair system. The genes mut H, mut L, mut S and mut U are responsible for the removal of the incorrect bases by an excision repair process. The mismatch repair enzymes initiate the removal of the incorrect base by nicking the DNA strand on both sides of mismatch. After all in a mismatch there are no defective bases. When

G may be mispaired with T, mismatch repair system needs some way to determine whether G-T is the correct base at a given site.

The mismatch repair system may be able to operate with or without methylation cues. In Fig. 3.3. DNA is nicked at GATC sequences. The mismatch is recognized by mut L and Mut S genes. The product of mut H gene nicks the DNA, the product of mut U, DNA helicase, unwinds the nicked region. DNA polymerase I and ligase fill in the patch. Thus the patch is repaired by mismatch repair mechanism and progeny strand is methylated (methylation play key role in control of DNA function).

The repair system makes this distinction by identifying the template strand with original nucleotides and newly synthesized strand with errors. This distinction is based on pattern of methylation in newly replicated DNA. Addition of methyl group to bases in DNA is called methylation where methylated DNA is found, transcription is stopped. e.g. methylated CPG dinuocleotides bind specific proteins that prevents transcription. In *E. coli*, A in CATC sequence is methylated. Thus time interval occurs during which template strand is methylated and nearly synthesized strand is unmethylated. The mismatch repair system uses this difference in methylation state to excise the mismatched nucleotide and replace it with the correct nucleotide GC by using the methylated parental strand of DNA as template.

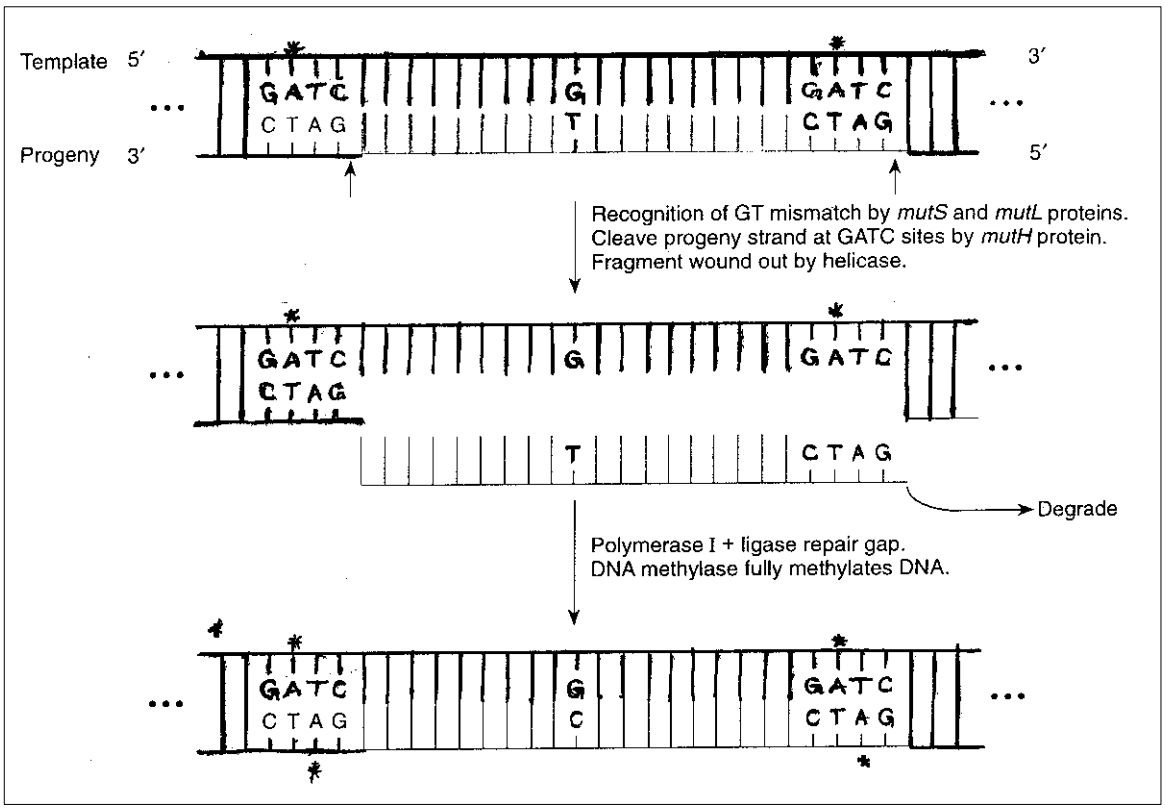

Fig. 3.3 A model of mismatch repair. **Asterisks** show the methylated adenines in the GATC sequences. The mismatched progeny section is removed by *mut*H and helicase after *mut*S and *mut*L recognise the mismatch. The patch is repaired and the progeny strand is methylated.

3.2.4 Post replicative repair (Recombinational Repair)

When DNA polymerase III encounters certain damage in *E. coli* **e.g**. thymine dimers, it can't proceed. It restarts after dimer, leaving a gap in the nascent strand, opposite the dimer in the template strand. The damaged DNA is repaired by recombination dependent repair process mediated by *E. coli* Rec A gene product. During post-replication repair, the Ree A protein, binds to the single strand of DNA at the gap and mediates pairing with the homologous segment of the sister double helix. The gap opposite the dimer is filled with homologous DNA strand from the sister DNA molecule (Fig. 3.4). Since the repair takes place at a gap created by the failure of DNA replication, the process is called postreplicative repair. Postreplicative repair is sometimes called recombinational repair.

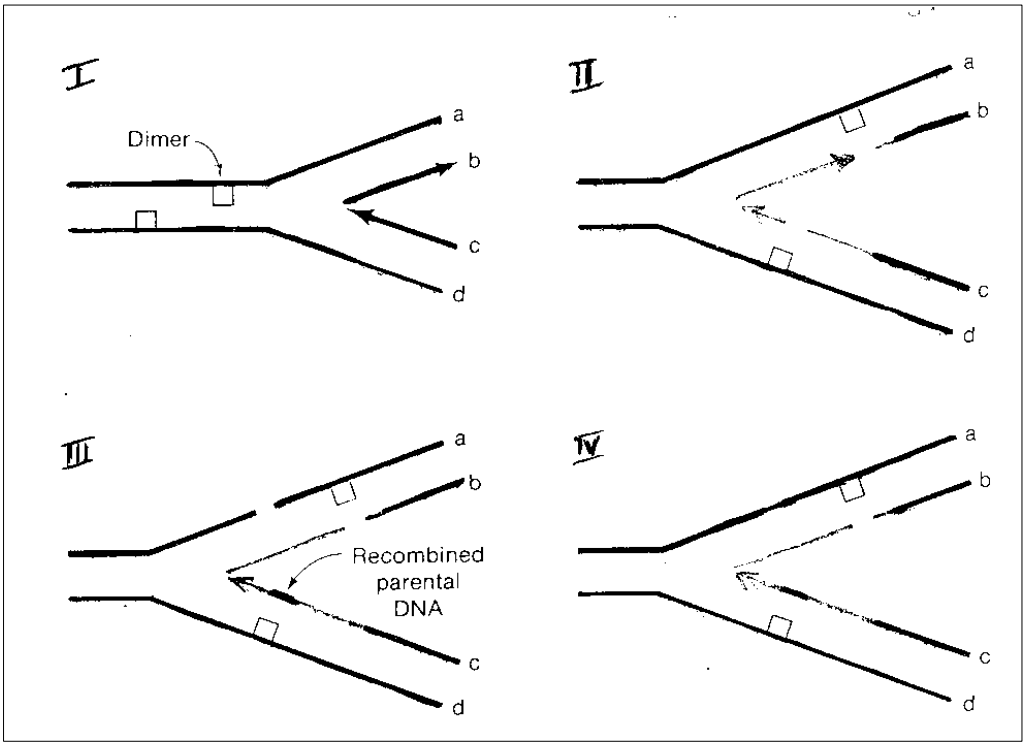

Fig. 3.4 Recombination repair **I.** A molecule containing two thymine dimers (red boxes) in strands **a** and **d** is being replicated. **II**. By postdimer initiation, a molecule is formed whose daughter strands **b** and **c** have gaps. If repair does not occur, in the next round of replication, strands **a** and **d** would yield gapped daughter strands, and strands **b** and **c** would again be fragmented. **III.** A segment of parental strand is excised and inserted into strand **c. IV**. The gap in strand **b** is similarly filled in by repair synthesis. Such **a** DNA molecule would probably engage in a second exchange in which a segment of **c** would fill the gap in **b**.

DNA synthesized after irradiation is shown thin. Heavy and thin lines are used for purposes of identification only (Microbial Genetics by David Freifelder).

3.2.5 SOS Repair

The DNA repair systems described so far are quite accurate. When DNA of *E. coli* cells is heavily damaged by mutagenic agents such as u.v. light, the cells take some drastic steps to survive. Then they go through a so called SOS response (save our souls) during which a whole battery of DNA repair, recombination and replication proteins are synthetized. Although, the proteins encoded by umu C, umu D genes, allow DNA replication to proceed across damaged segments of template strands, even though nucleotide sequence in damaged region can't be accurately replicated. Thus, it is error-prone repair system, which eliminates gaps in the newly synthesized strand, opposite damaged nucleotides in the template strands and in so doing, increases the frequency of replication errors. Since, it is error prone repair system, mutation rates increase sharply by this repair mechanism.

Recent research on DNA repair mechanisms indicates, many new repair processes remain to be understood. These results suggest that we have much to learn about the mechanisms that safeguard the integrity of our genetic information.

3.3 SUMMARY

Mutations occur in organisms either spontaneously or induced by mutagenic agents in the environment. DNA repair mechanisms in *E. coli* cells are well-characterized i.e., light dependent repair or photoreactivation, excision repair, mismatch repair, post-replication repair, error-prone repair (SOS). In protoreactivation, thymine dimers are separated by photolyase enzyme in the presence of light. Excision repair is a process in which a damaged section of a strand of DNA is removed and the gap is filled by DNA polymerase I.

In postreplicative repair, the damaged DNA is repaired by recombinant dependent repair process. The Rec A protein plays key role in it. A single strand from the undamaged duplex is used to fill the gap in the damaged duplex. When DNA of *E. coli* cells is heavily damaged, they take drastic steps to survive through SOS response, which is error prone repair system. The above mechanism of DNA repair, enzymes involved are briefly described here.

3.4 MODEL QUESTIONS

1. Describe the mechanisms of DNA repair you have studied.
2. Differentiate between light dependent and other types of DNA repair mechanisms.

3. Write short notes on:
   a) Thymine dimers
   b) Photoreactivation
   c) Excision Repair
   d) Post-replicative repair

3.5 REFERENCE BOOKS

1. **Instant Notes Molecular Biology** by Turner, P.C., McLennan A.D. Bates & MRH White, Viva Books Ltd., 2001.
2. **Principles of Genetics** by Tamarin, 2000.
3. **Principles of Genetics** by Peter Snustad and M.J. Simmons, 2003.
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M.Sc. BOTANY (Final)

Paper- VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-IV

Lesson 4
Gene Expression

4.1 INTRODUCTION

The ability of living organisms is to grow and reproduce They synthesize many different molecules/enzymes to obtain energy for growth. Proteins contain amino acids and each protein consists of either a single chain of amino acids or two or more chains called a polypetide. The amino acid sequence of each polypeptide is controlled by one gene. Phenotype of an organism is produced by combined effects of genes. The RNA genomes of smallest virus like MS2 phage contain only four genes, Bacteria like *E. coli* have about 4000 genes and humans have 30,000 to 40,000 genes. In this chapter, the gene expression on the mechanisms by which genes direct the synthesis of their products namely RNAs and proteins are described.

According to the central dogma of molecular biology, genetic information usually flows from DNA to protein via RNA. It involves two steps: 1) transcription, the transfer of the genetic information from DNA to RNA. (2) translation, the transfer of information from RNA to protein.

Thus genetic information always flow from DNA to RNA (Fig. 4.1) except in some viruses from RNA to DNA (during the conversion of genomes of RNA tumor viruses to integrated form of proviral DNA in the host genome). This process of transfer of genetic information from RNA to DNA can be understood from the study of Reverse genetics a modified central dogma. The expression of genetic information occurs in two steps (Fig. 4.2); transcription and translation. The mechanism of transcription is described below:

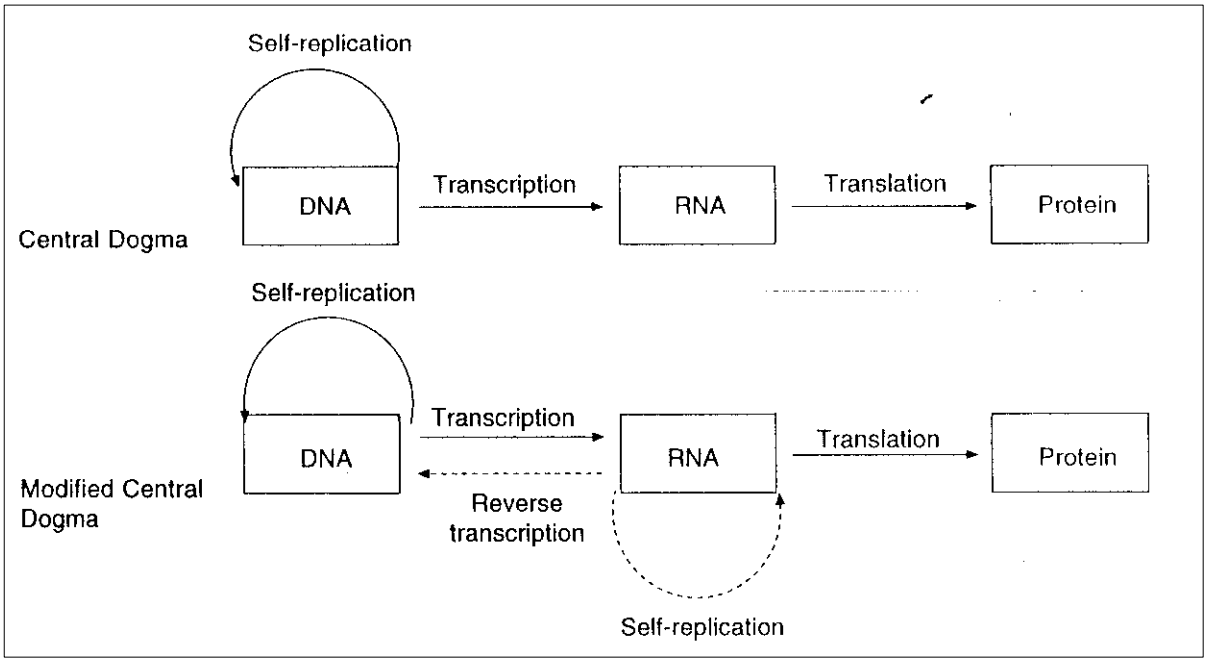

Fig. 4.1 Central dogma for protein synthesis

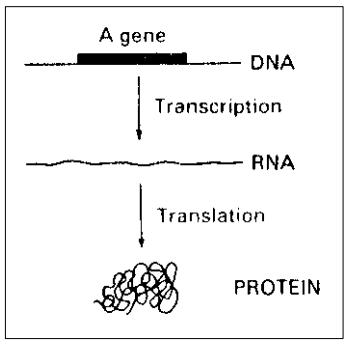

Fig. 4.2 The two stages of gene expression

4.2 TRANSCRIPTION (in prokaryotes)

During transcription, one strand of DNA of a gene is used as template to synthesize a complementary strand of RNA, called the gene transcript. Using DNA, RNA as a template strand,

the RNA polymerase enzyme initiates transcription. The synthesis is 5' → 3' direction only. The process of transcription can be divided into three stages: (1) initiation of a new RNA chain, (2) elongation of the chain, (3) termination of transcription and release of the nascent RNA molecules (Fig. 4.3).

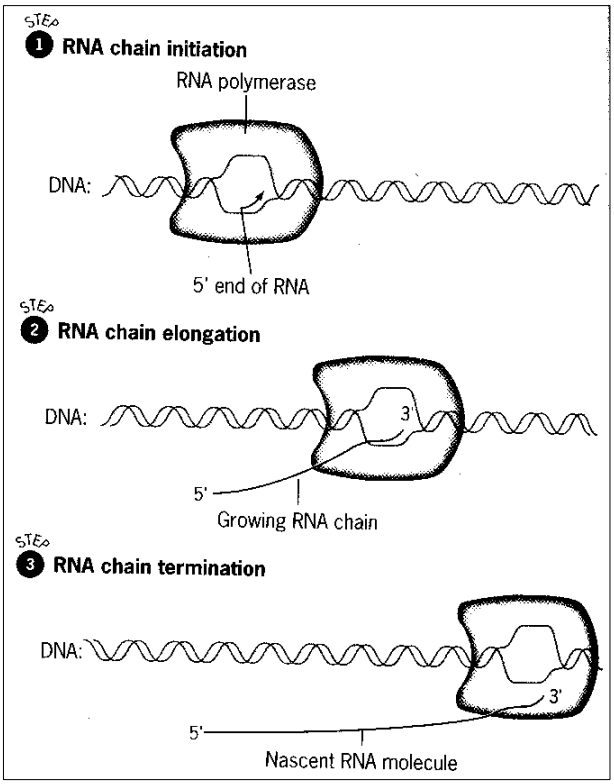

Fig. 4.3 The three stages of transcription: initiation, elongation and termination →

4.2.1 RNA polymerases

In *E. coli* only one RNA polymerase is involved in transcription. It has mol. wt. of 480,000 and five polypeptides. The holoenzyme has α_2 β B' and σ (Fig. 4.3). The sigma (σ) factor is involved in the initiation of transcription. After initiation of the chain, sigma factor is released and chain elongation is catalyzed by the core enzyme (α_2, αB'). in eukaryotes three RNA polymerases are involved. The key features of the three eukaryotic RNA polymerases are summarized in Table 1. All three eukaryotic RNA polymerases require the assistance of other proteins called transcription factors to initiate the synthesis of RNA chains in eukaryotes.

Table 1. Characteristics of the three RNA polymerases of Eukaryotes

Enzyme	**Location**	**Products**
RNA polymerase I	Nucleolus	Ribosomal RNAs, excluding 5S rRNA
RNA polymerase II	Nucleolus	Nuclear pre-mRNAs
RNA polymerase III	Nucleolus	tRNAs, 5S rRNA and other small nuclear RNAs

Initiation of Transcription: It involves three steps: (1) binding of RNA polymerase to a promoter region in DNA, (2) the localized unwinding of the two strands of DNA by RNA polymerase, (3) the formation of phosphodiester bonds between the ribonucleotides in RNA chain. The Nucleotide sequences preceding the initiation are referred as upstream sequences (+) and following the initiation site are referred as downstream sequences (-) (Fig. 4.4).

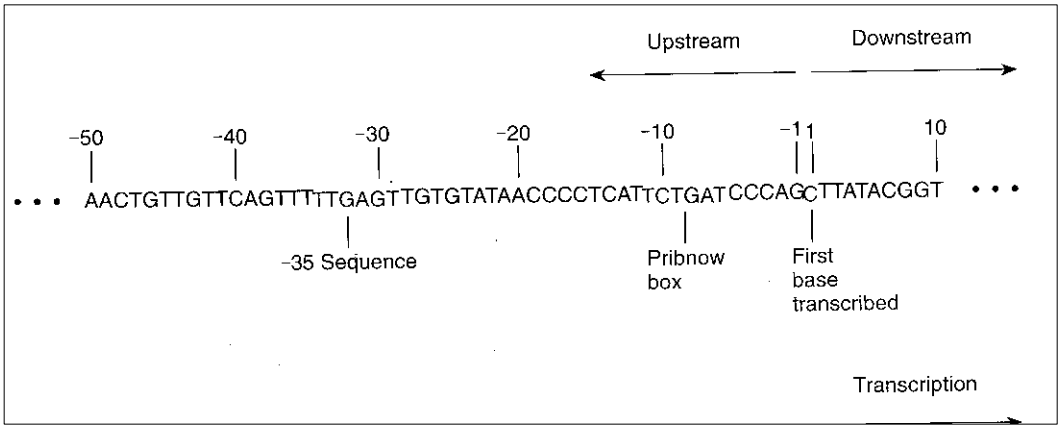

Fig. 4.4 A promoter in the *E. coli ori*C region with the –35 and –10 sequences shown as well as the first base transcribed. The upstream and downstream directions are indicated.

4.2.2 Promoter sequences

The region of DNA, where RNA polymerase associates before transcription is known as promoter. Two short sequences within these promoters are conserved. They occur at about 10 and 35 nucleotide pairs before transcription initiation site. The nucleotide sequences that are present in such conserved genetic elements are called **Consensus sequences**. The - 10 consensus sequences is TATAAT, the - 35 consensus sequence is TTGACA. The sigma subunit binds to - 35 sequences, thus it is called the recognition sequence. A : T rich-10 sequence facilitates having only two hydrogen bonds for easy unwinding of DNA. The distance between -35 and -10 sequences is highly conserved in *E. coli* promoters. It is never less than 15 or more than 20 nucleotide pairs in length (Fig. 4.4).

4.2.3 Elongation of RNA chain

The RNA polymerase molecule continuously unwinds the DNA double helix, ahead of polymerization site and rewinds the complementary DNA strands behind the polymerization site, as it moves along the double helix (Fig. 4.5). The average length of transcription bubble is 18 nucleotide pairs, and about 40 nucleotides are incorporated into the growing RNA chain per second.

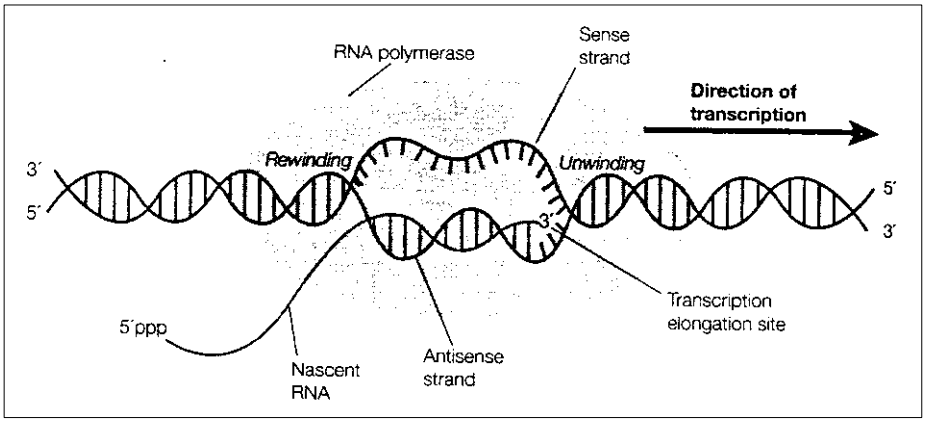

Fig. 4.5 Schematic structure of the transcription bubble during polymerization

4.2.4 Termination of RNA chain

Termination of RNA chain occurs when RNA polymerase recognizes a termination signal/ termination sequences. There are two types of transcription terminators in *E. coli*. One type results termination, only in the presence of a protein called **rho**, therefore, such termination sequences are called **rho-dependent terminators** (Fig. 4.6). The mechanism by which the dependent termination of transcription occurs is still uncertain. The other type results in the termination of transcription without the involvement of rho, such sequences are called rho-independent terminators (Fig. 4.7).

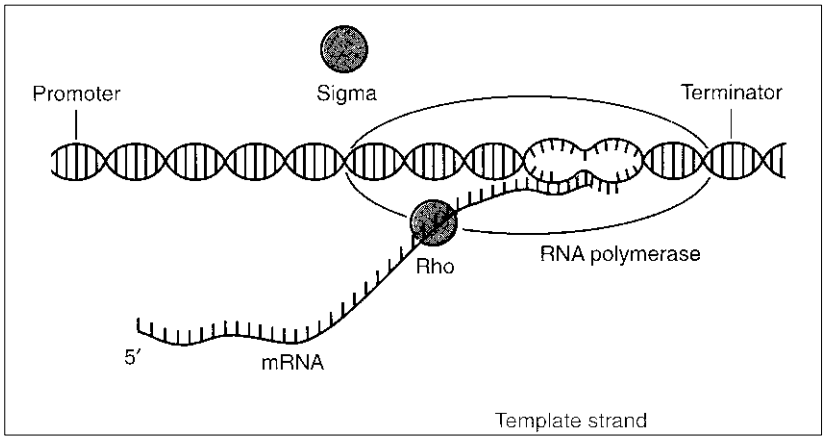

Fig. 4.6 Transcription overview and RNA polymerase molecules. RNA polymerase is transcribing near the promoter. The rho factor is shown on the newly formed RNA and the sigma factor is shown nearby, detached from the core polymerase.

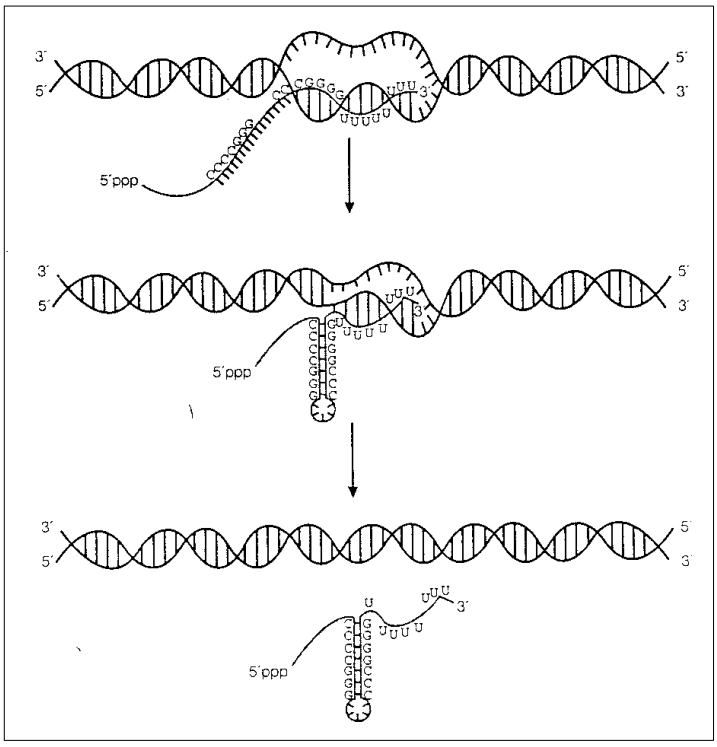

Fig. 4.7 Schematic diagram of rho-independent transcription termination

Rho-independent terminators contain G: C rich region (Fig. 4.7) followed by six or more A: T base pairs. The single stranded RNA form hairpin-like structure after the synthesis and retard the movement of RNA polymerase along the DNA causing a pause in the chain extension.

4.3 TRANSCRIPTION (in Eukaryotes)

Although overall process of RNA synthesis is similar in prokaryotes and eukaryotes, the process is considerably more complex in eukaryotes. Because in eukaryotes, RNA synthesized in the nucleus is transported to the cytoplasm for translation on ribosomes; whereas it is a coupled transcription and translation in prokaryotes in which, while mRNA is being synthesized, protein synthesis occurs on the mRNA strand (Fig. 4.8). In contrast many of the eukaryotic transcripts have been characaterized that contain the coding region of a single gene (monogenic). Once it is formed it undergoes three major modifications as shown in (Fig. 4.9).

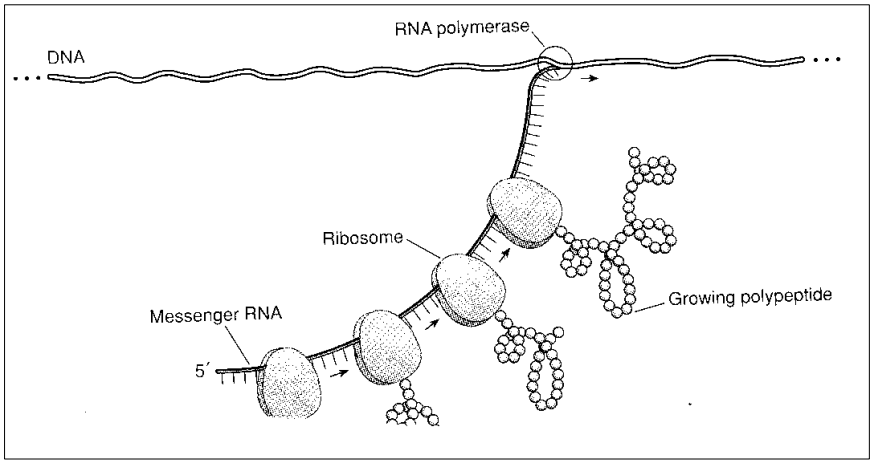

Fig. 4.8 In prokaryotes, translation of messenger RNA by ribosomes begins before transcription is completed. Ribosomes attach to the growing mRNA strand when the 5' end becomes accessible. They then move along the RNA as it elongates. When the first ribosome moves from the 5' end, a second ribosome can attach and so on.

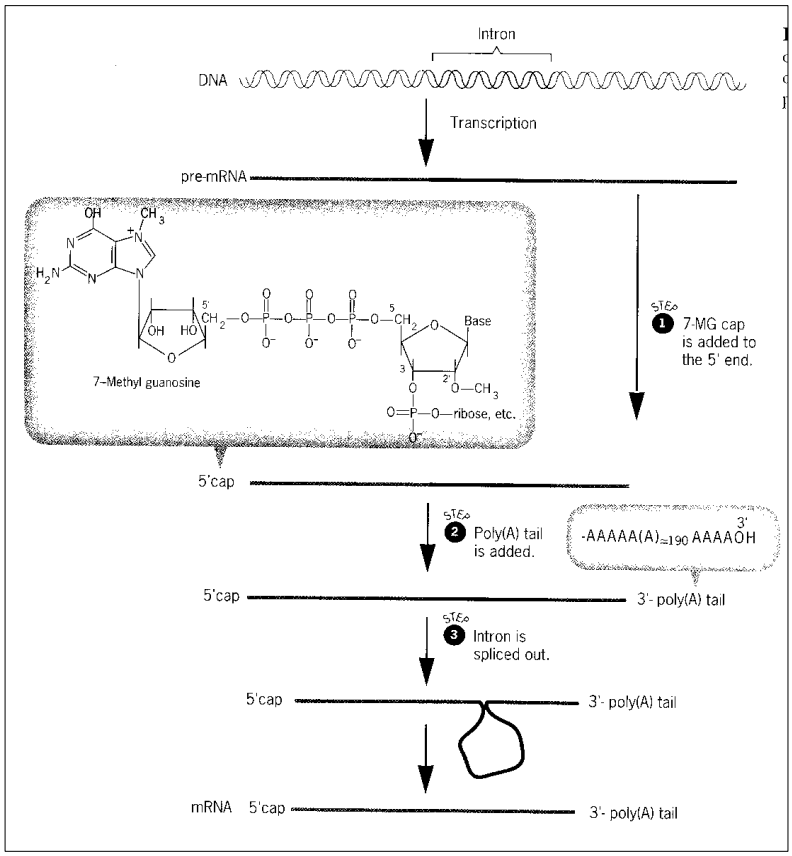

Fig. 4.9 In eukaryotes, most gene transcripts undergo three different types of post-transcriptional processing

4.3.1 RNA Processing

1. 7-methyl guanosine caps are added to the 5' end of the primary transcripts.

2. Poly (A) tails are added to the 3' end of the transcripts.

3. Intron sequences are spliced out of transcript.

In eukaryotes, the primary transcript in a nucleus is called heterogeneous nuclear RNA (hn RNA) due to large variation in the sizes of RNA molecules present. Major portions of it contain non-coding regions called introns which are spliced out. The average half-life of a gene transcript in eukaryote is about five hrs, but it is less than five minutes in *E. coli*. The stability of mRNA is provided due to 5' cap end of mRNA which helps to protect the growing RNA chains from degradation. The poly (A) tails of eukaryotic mRNAs enhance the stability of mRNA and plays important role in the transport from the nucleus to the cytoplasm.

Transcription factors must bind to a promoter region in DNA and form initiation complex before RNA polymerase initiate transcription. Different promoters and transcription factors are utilized by the RNA polymerases I, II and III in case of Eukaryotes.

4.3.2 Promoters recognized by RNA polymerase II

It consists of short conserved elements, upstream from conserved transcription start point. The element close to the transcription start site (+1) is called the TATA box; It has the consensus sequence TATAAAA and is present at about -30 positive. The second conserved element is CAAT box at -80 position and has consensus sequence GGCCAATCT. Two other conserved elements GC box, GGGCGG and octamer box ATTTGCAT often are present in promoter region recognized by RNA polymerase II (Fig. 4.10).

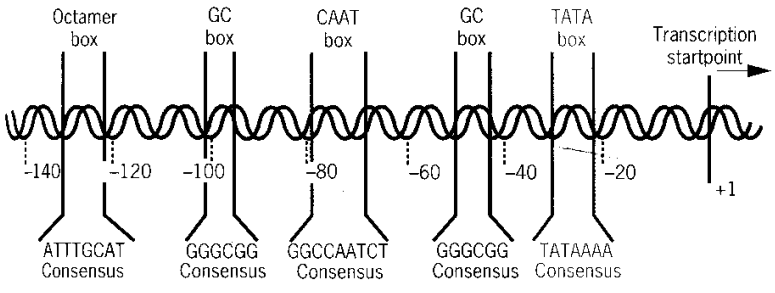

Figure 4.10 Structure of a promoter recognized by RNA polymerase II. The TATA and CAAT boxes are located at about the same positions in the promoters of most nuclear genes encoding proteins. The GC and octamer boxes may be present or absent; when present, they occur at many different locations, either singly or in multiple copies. The sequences shown here are the consensus sequences for each of the promoter elements. The conserved promoter elements are shown at their locations in the mouse thymidine kinase gene.

The initiation of transcription by RNA polymerase II requires the assistance of several basal transcription factors TF II X (Transcription factor for polymerase II X is a letter identifying the individual factor). TF II D is the first to interact with the promoter. Later several transcription factors like A B F and E joins the initiation complex in stepwise manner, while binding to the DNA downstream from the transcription start point (Fig. 4.11).

Similar transcription factor complexes are required to initiate transcription by RNA polymerase I and III. The promoters of genes transcribed by polymerases I and III are quite different from the promoters utilised by RNA polymerase II.

4.3.3 RNA chain elongation

Once eukaryotic RNA polymerases have been released from their initiation complexes, they catalyze RNA chain elongation by same mechanism as the RNA polymerase of prokaryotes. Early in elongation process, 5' ends of eukaryotic pre-mRNAs are modified by addition of 7-methylguanosine (7-MG) caps. These caps help to protect the growing RNA chain from degradation by nucleases as mentioned earlier during the phenomenon of RNA processing.

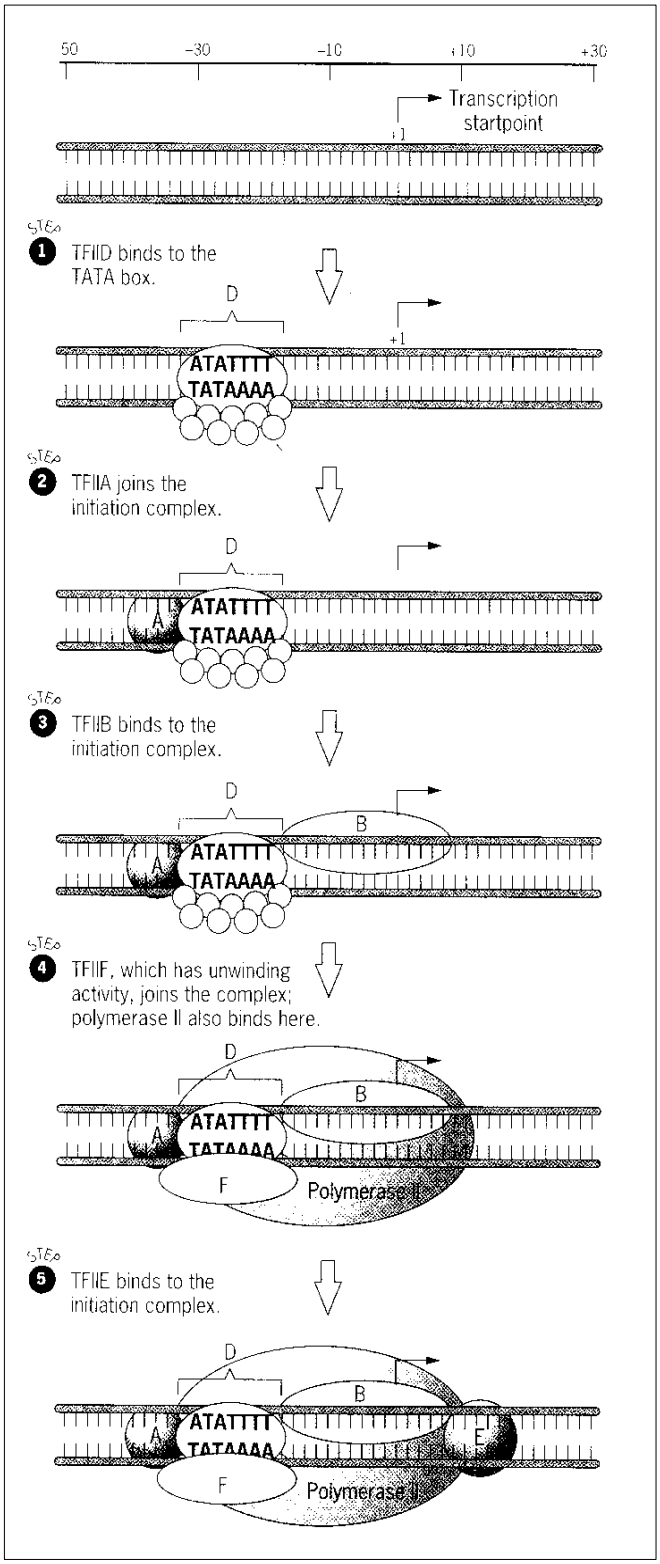

Fig. 4.11 The initiation of transcription by RNA polymerase II requires the formation of a basal transcription initiation complex at the promoter region. The assembly of this complex begins when TFHD, which contains the TATA-binding protein (TBP), binds to the TATA box. The other transcription factors and RNA polymerase II join the complex in the sequence shown.

4.3.4 RNA chain termination

The transcription termination events occur at multiple sites located at 1000 to 2000 nucleotides downstream from the site that become 3' end of the chain. After cleavage, the enzyme polymerase adds poly A tails, to the3' end of the transcripts. The addition of poly A tails to eukaryotic mRNAs is called polyadenylation. The poly(A) tails of eukaryotic mRNAs enhance their stability and play an important role in their transport from the nucleus to the cytoplasm.

4.3.5 RNA Editing

According to the central dogma of molecular biology, genetic information flows from DNA via RNA to protein during gene expression. Normally, the genetic information is not altered in the mRNA intermediary. However, the discovery of process, RNA editing (Fig. 4.12) has shown that exceptions do occur. RNA editing processes / changes the information content of gene transcript by two ways: (1) by changing the structures of individual bases, and (2) by inserting or deleting uridine monophosphate residues.

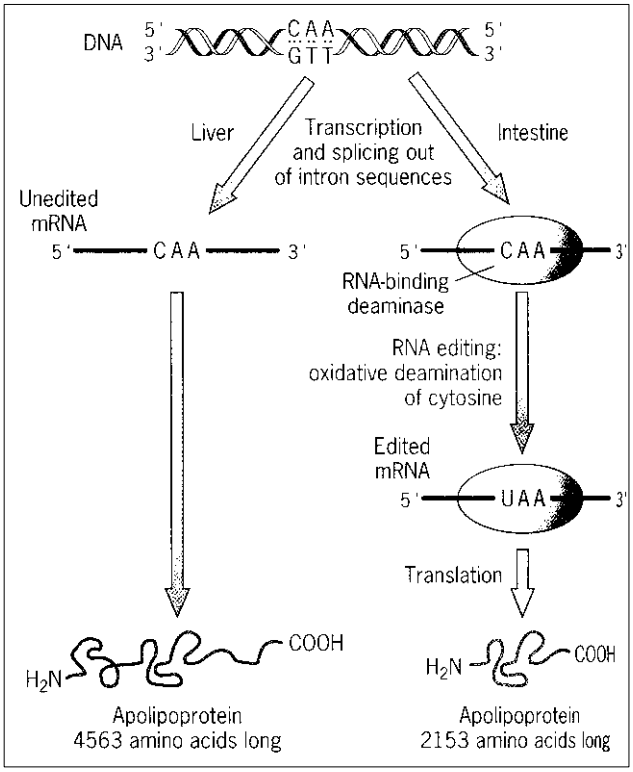

Fig. 4.12 Editing of the apolipoprotein-B mRNA in the intestines of mammals.

A more complex type of RNA editing occurs in the mitochondria of trypanosomes (Flagellated protozoa, causing sleeping sickness in humans). RNA editing process is mediated by guide RNAs transcribed from distinct mitochondrial genes. Why do these editing processes occur? One view is RNA editing was common in ancient cells where reactions have been catalyzed by RNA molecules instead of proteins. Another view is that RNA editing is a primitive mechanism for altering patterns of gene expression. Thus, RNA editing plays role in the expression of genes in mitochondria of trypanosomes and plants.

4.3.6 Self splicing of rRNA precursor (Removal of intron sequences)

Self splicing by RNA was discovered by Thomas Eech and his colleagues in 1982 including Altman who showed that RNA can have catalytic properties. They found introns can be removed without the help of proteins. Therefore, an introus is acting as an enzyme. We call an RNA with enzymatic properties a ribozyme. Self splicing of rRNA precursor in *Tehrahymena* is shown in Fig. 4.13. It displaces G at 3' side of intron, reconnecting RNA with u-u conncection and releases the intron. Self splicing of this type is done by **group I introns**. Thus the spliced out intron is capable of continued enzymatic activity using its own sequence as a template and act on RNAs. It can act like endonuclease (splitting RNA strands), polymerase, transferase or ligase. Therefore, introns work like molecular scissors as suggested by Walter Gilbert. The ability of RNA to act as an enzyme has led to another advancement in our thinking, whether RNA or DNA is the first genetic material of living organism.

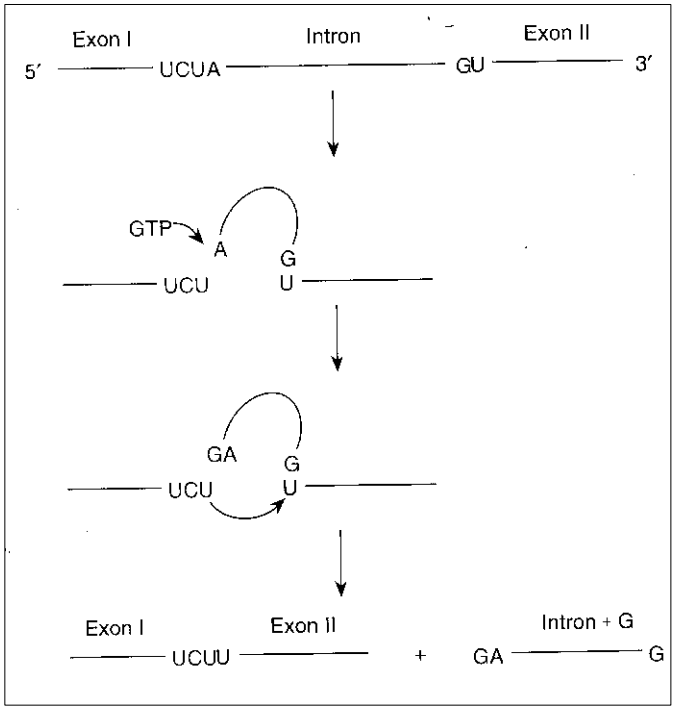

Fig. 4.13 Self-splicing of an rRNA precursor in Tetrahymena. An external GTP is required. Two bond transfers result in a shortened RNA and a free intron.

Group II introns use a different mechanism of splicing. Nuclear mRNAs have their introns removed by way of a lariat structure. Here introns are removed with the help of protein-RNA complex called a **spliceosome**. The splicing apparatus in eukaryotic mRNAs consists of several components called small nuclear ribonucleoproteins, **snRNPs** named as snurps. Five of these particles take part in splicing each composed of one or more proteins and small RNA molecule. Hence, a spliceosome removes an intron (Fig. 4.14). Thereby the splicing mechanism provides evidence in the evolution of Eukaryotes.

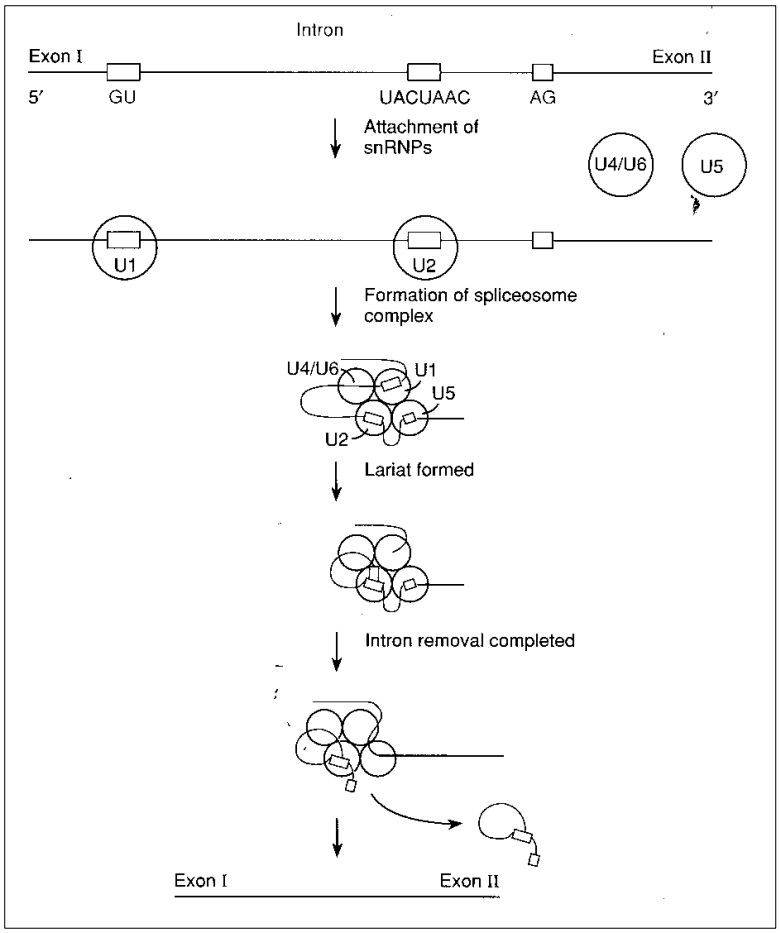

Fig. 4.14 Spliceosome removes an intron. The spliceosome complex forms, leading to lariat formation and intron removal. The role of the U5 and U4/U6 snRNP, is presently not known.

Both the views, introns - early and the introns - late may be correct. Introns arose early, were lost by prokaryotes, in which small genomes and efficient DNA replication were priorities and later they evolved in eukaryotes to produce the **exon shuffling** as suggested by Gill bert.

Thus RNA editing and self splicing of RNA are important in understanding the significance of molecular Biology in the Evolutionary mechanisms.

4.4 SUMMARY

The central dogma of molecular biology is that the genetic information flows from DNA to protein during gene expression through two steps: 1) Transcription and Translation. Transcription

involves the synthesis of an RNA transcript, complementary to sense strand of DNA. mRNA synthesis, utilizes only one DNA strand of a gene as template, unlike DNA replication which utilizes both the strands during the synthesis of daughter DNA molecule. The synthesis of mRNA is 5'-3' direction.

The process can be divided into three stages: (1) initiation of a new RNA chain, (2) elongation of the chain, (3) termination and release of RNA molecule. The mechanism of transcription is described in which the role of RNA polymerase enzyme and promoters is described. The differences between prokaryotes and eukaryotes, in the process of transcription are mentioned. The phenomenon of RNA editing and self-splicing of RNA was briefly described keeping in view of its evolutionary significance.

4.5 MODEL QUESTIONS

1. Explain the differences between RNA polymerases involved in Eukaryotes and Prokaryotes. List three ways in which mRNAs of eukaryotes differ from the mRNAs of prokaryotes.
2. Describe the mechanism of transcription while mentioning the differences between prokaryotes and eukaryotes.
3. Write short notes on:
   a) Promoters
   b) Self splicing of rRNA
   c) Transcription bubble
   d) RNA editing
   e) RNA polymerases

4.6 REFERENCE BOOKS

1. **Instant Notes Molecular Biology** by Turner, P.C., McLennan A.D. Bates & MRH White, Viva Books Ltd., 2001.
2. **Principles of Genetics** by Tamarin, 2000.
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Prof. T. N. MARY

M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-IV

Lesson 5

Gene Expression - Translation and the Genetic code

5.0 **OBJECTIVE:**

In the earlier lesson, the transfer of genetic information from DNA to RNA in the process of transcription was discussed. In this lesson, we study the process how genetic information stored in mRNAs is used in synthesis of polypeptide gene products. The protein synthesis and important features of genetic code are explained below:

5.1 INTRODUCTION

In protein synthesis (Fig. 5.1) genetic information stored in the sequence of nucleotides in mRNA is translated into a form of protein, according to the specifications of the genetic code. Thus protein synthesis requires a large number of macromolecules: 1) 3-5 RNA molecules, 2) at least 20 amino acid activating enzymes present in each ribosome 40 to 60 tRNA moles, 3) numerous soluble proteins involved in polypeptide chain initiation, elongation and termination etc.

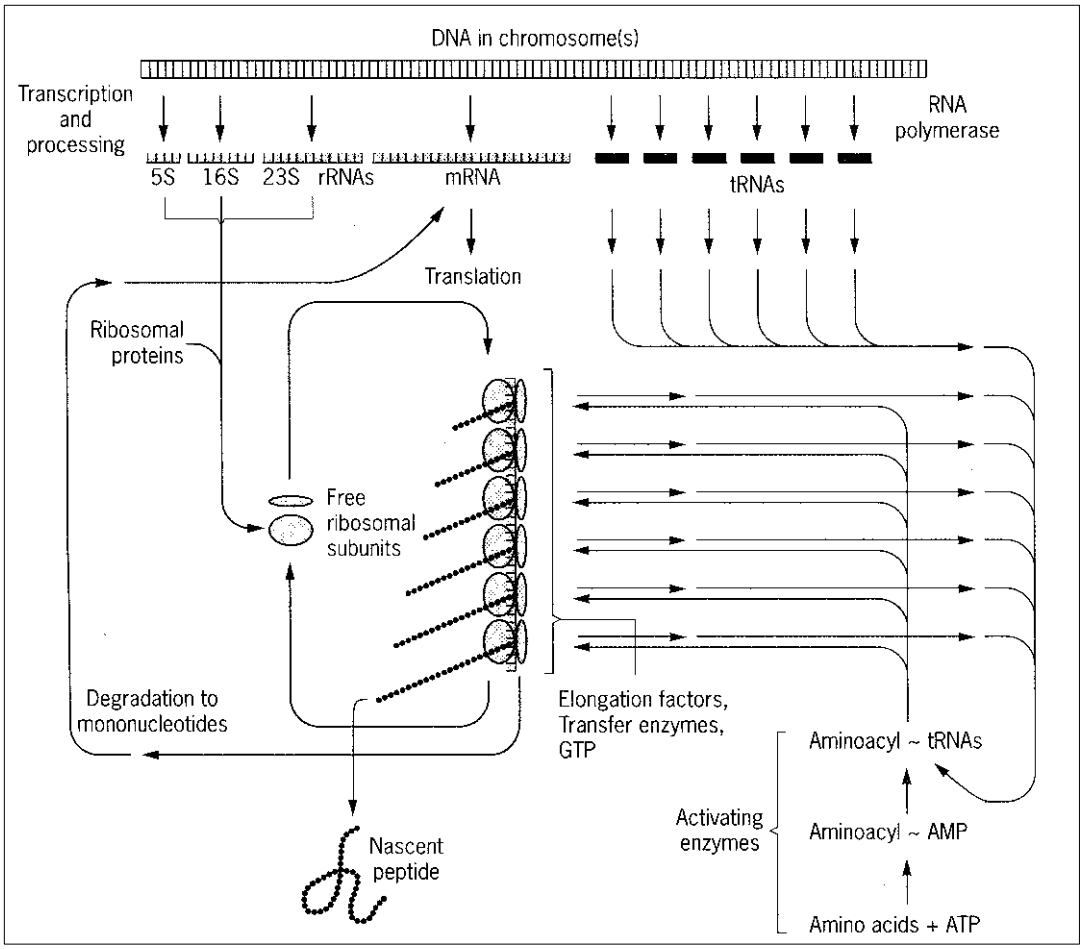


Fig. 5.1 Overview of protein synthesis. The sizes of the rRNA molecules shown are correct for bacteria; larger rRNAs are present in eukaryotes. For simplicity, all RNA species have been transcribed from contiguous segments of a single DNA molecule. In reality, the various RNAs are transcripts of genes located at different positions on from one to many chromosomes.

Translation occurs on ribosomes, which are complex macromolecular structures located in the cytoplasm. It involves three types of RNA, i.e., mRNA, rRNA, tRNA, all transcribed from DNA templates. Ribosomes are work benches in protein synthesis having approximately half protein and half RNA. They are composed of two subunits, one large and one small 50s, 30s respectively named Svedberg units (after the Scientist Svedberg) according to their semidementation rates during centrifugation. Although the size and composition of ribosomes vary in prokaryotes and eukaryotes with regard to type of rRNA and ribosomal protein as shown in Fig. 5.2, the outer cell three dimensional structure of the ribosome is same in all organisms. Masayasu

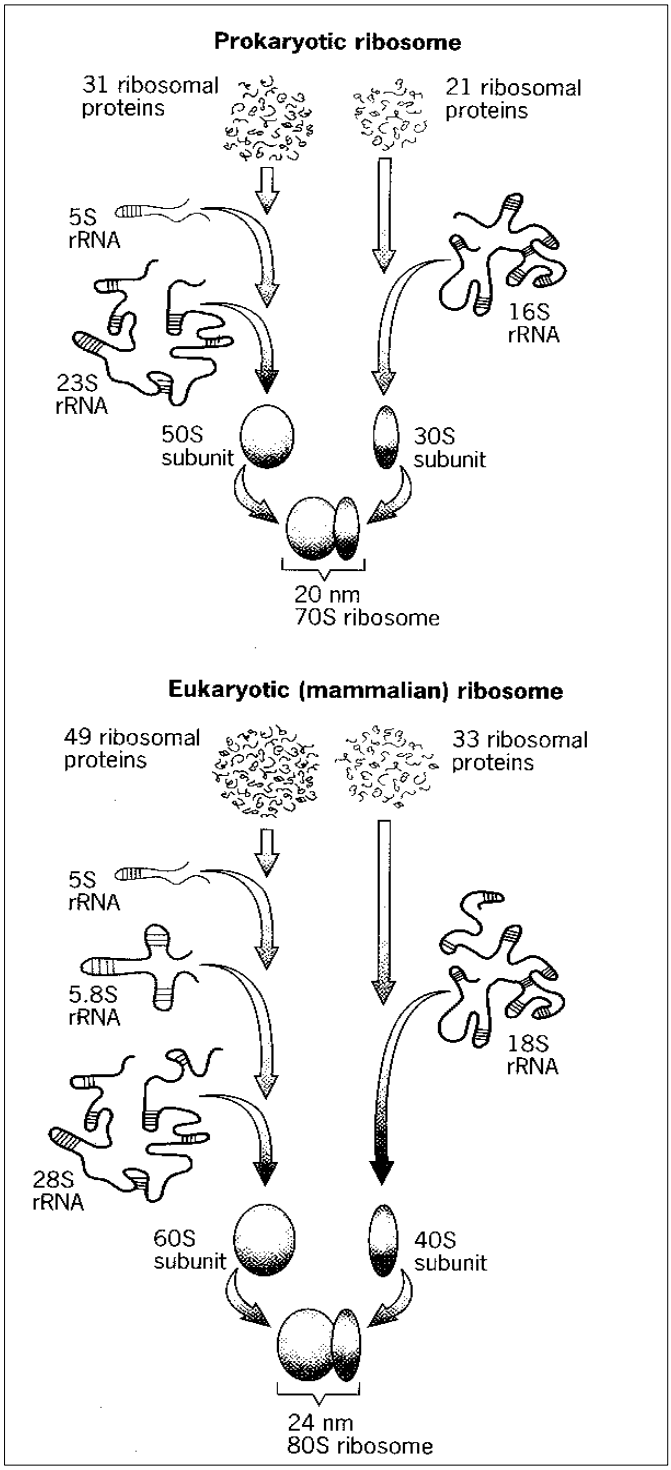


Fig. 5.2 Prokaryotic ribosome and Eukaryotic (mammalian) ribosome.

Nomura and his colleagues were able to study the ribosomal subunits of *E. coli*. In Eukaryotes rRNA synthesis occurs in the nucleolus. In *E. coli*, rRNA gene undergoes cleavage to produce 5S, 16S, 23S rRNAs plus 4S tRNA molecle. In mammals 5.8S, 18S, 28S rRNAs are cleaved from 45S rRNA precursor (Fig. 5.3). The genes of rRNA of eukaryotes are present in the nucleolar organizing region of chromosomes.

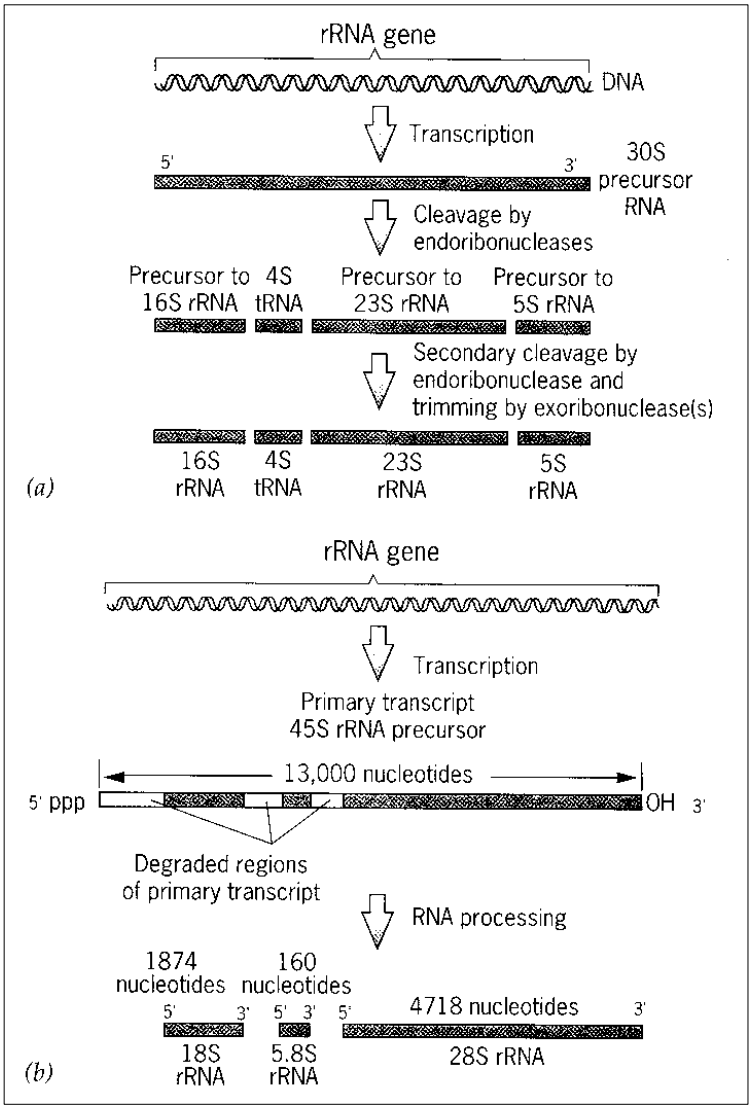


Fig.5.3 Synthesis and processing of (a) the 30S rRNA precursor in *E. coli* and (b) the 45S rRNA precursor in mammals

Although the ribosomes provide many of the components required for protein synthesis, rRNA molecules act as adaptor molecules. There are one to four tRNAs for each of the 20 amino acids.

Transfer RNA

Each cell contains many different types of tRNA molecules. Transfer RNAs are the adapter molecules that read the nucleotide sequences of mRNA transcript and convert it into a sequence of amino acids. Transfer RNA molecules are relatively small, mostly between 74 and 95 nucleotides, for different molecules in different species. Almost every tRNA molecule in every organism can be folded into a base paired structure referred to as a clover leaf (Fig. 5.4). Holly (1965) and his colleagues determined the complete sequence of alanine transfer RNA. This structure is made up of the following components.

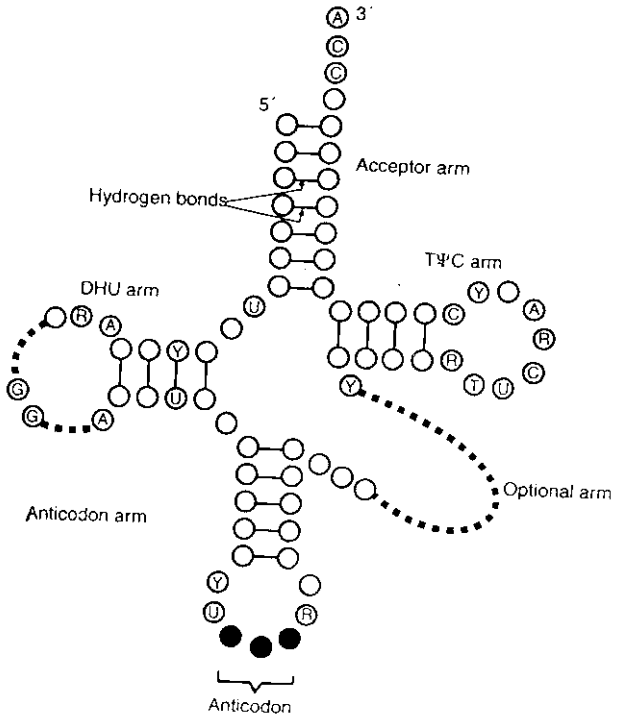


Fig. 5.4 Cloverleaf model of tRNA

1. The acceptor arm formed by a series of usually seven base pairs between nucleotides at the 5′ and 3′ ends of the molecule. During protein synthesis, an amino acid is attached to the acceptor arm of the tRNA.

2. The D or DHU arm, so named because the loop at its end almost invariably contains the unusual pyrimidine dihydrouracil.

3. The anticodon arm plays the central role in decoding the biological information carried by the mRNA.

4. The extra, optional or variable form that may be a loop of just 3-5 nucleotides, or a much larger stem loop of 13-21 nucleotides with up to 5 base pairs in the stem.

5. The TΨC arm, named after the nucleotide sequence T, Ψ, C (Ψ = nucleotide containing pseudouracil, another unusual pyrimidine base), which its loop virtually always contain.

In both prokaryotes and eukaryotes, tRNAs are transcribed initially as precursor tRNAs, which are subsequently processed to release the mature molecules. A pre-tRNA molecule is processed by a combination of different ribonucleases that make specific cleavages at the 5′ (Rnase P) and 3′ (Rnase D) ends of the mature tRNA sequence. Eukaryotic tRNA genes are also clustered, and in addition occur in multiple copies.

5.2 STEPS IN PROTEIN SYNTHESIS

(1) The first step in a protein synthesis is tRNAs are activated or charged by amino aeyl-tRNA synthetase.

amino acid + ATP

amino acyl - tRNA synthetase ↓ Activation

amino acid AMP + (p) ~ (p) released and binds with tRNA

amino acid m AMP + tRNA

amino acyl – tRNA synthetase ↓

amino acid tRNA + AMP Activated tRNA

(2) Activated tRNA recognize correct codon on mRNA.

(3) Thus they must have correct anticodon sequence and also must be recognized by the correct aminoacyl – tRNA syntheses.

(4) There are three tRNA binding sites on ribosome (Fig. 5.5). The **A** or amino acyl site binds with the incoming aminoaeyl t-RNA. The **P** or peptidyl site binds the tRNA to which the growing popypeptide is attached. The **E** or exist site binds the departy uncharged tRNA. These binding ites are on 50S subunit and mRNA is bound by 30S subunit.

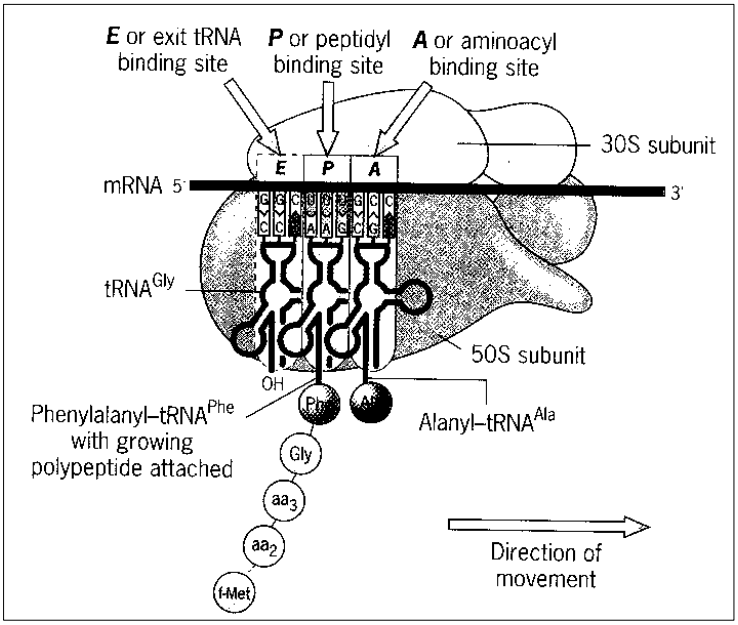


Fig. 5.5 70S ribosome

5.2.1 Inliation of Translation

The initiation process involves 30S subunit, tRNA, mRNA, protein initiation factors (IF-I, IF-2, IF-3) and one molecule of GTP. 50s subunit joins the complex to form 70s ribosome in the final step of initiation process (Fig. 5.6).

The synthesis of polypeptides is initiated by tRNA Met against initiation codon AUG. In prokaryotes mRNA contains a conserved 7 nucleotide complementary sequence. AGGAGG, upstream from initiation codon, called Shine - Dalgarno sequence, where 16S rRNA lies against the mRNA. This sequence is named after the Scientists, who discovered it. In the final step there is addition of 50S subunit to 30S initiation complex to produce 70S ribosome. The addition of 50s subunit requires energy from GTP and release of IF-I, IF-2.

In eukaryotes, the over all process is similar except, translation begins at AUG closest to 5' terminus of mRNA not at Shine Dalgarno sequence in prokaryotes. Amino group of methionyl tRNA Met is not formylated. If involves several soluble initiation factors. The entire initiation

complex moves from 5'→3' direction along mRNA searching for AUG codon. When AUG Triplet is found the 80s ribosome/mRNA/tRNA complex is ready to begin i.e., the next step of translation, is chain elongation.

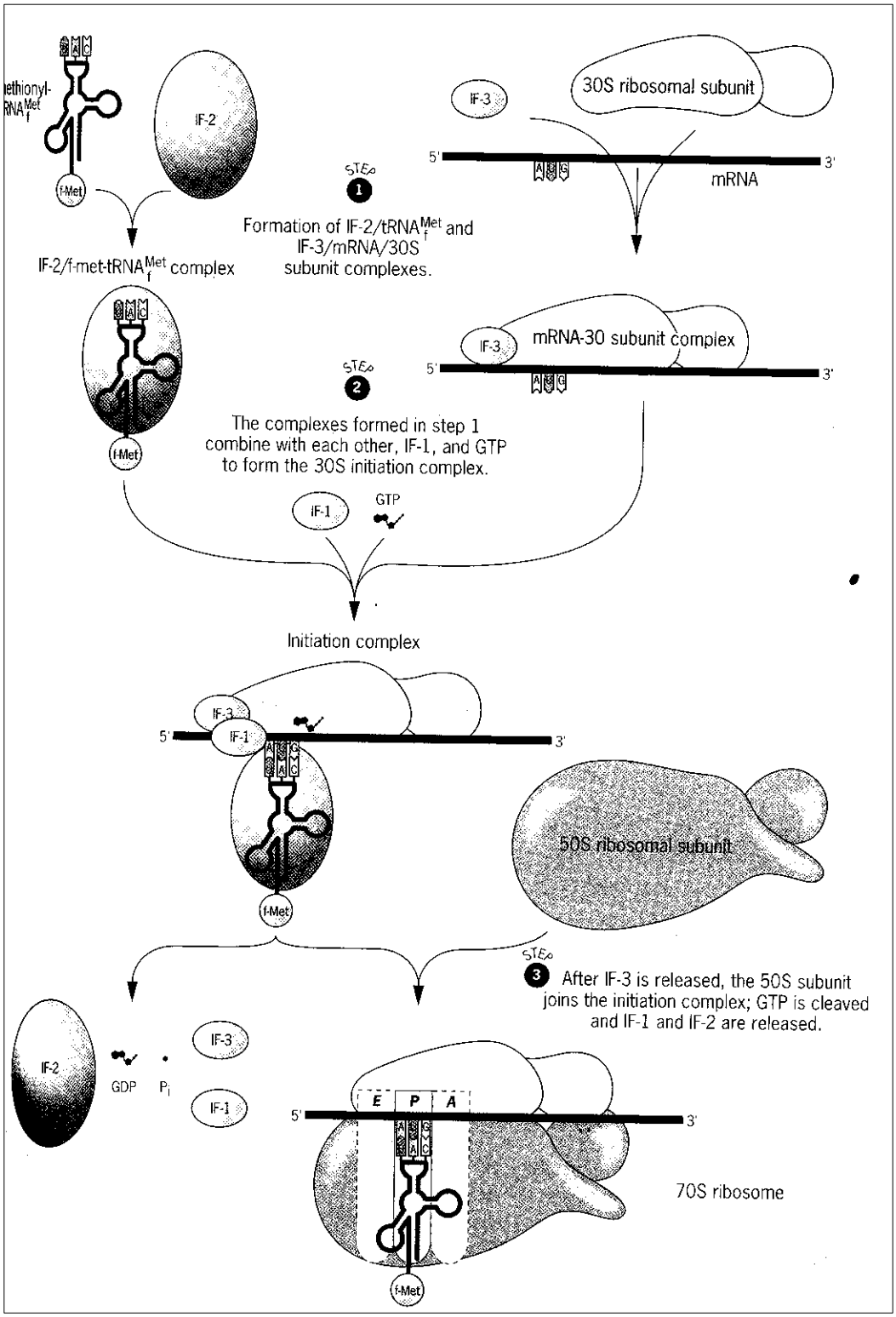


Fig. 5.6 The initiation of translation in *E. coli*

Table 1. Comparison between prokaryotic and eukaryotic translation

	Prokaryotes	**Eukaryotes**
Initiation codon	AUG, GUG, UUG	AUG
Initiation Aminoacid	N-formyl methionine	Methionine
Initiation tRNA	$tRNA_f^{Met}$	$tRNA_i^{Met}$
Initiation factors	IF1 IF2 IF3	Many IF factors
Elongation Factors	EF-Tu, EF-Ts	eEF I
Translocation Factor	EF G	eEF 2
Release Factors	RF 1, RF 2	eRF

5.2.2 Elongation of polypeptide chain

The process of elongation is basically same in both prokaryotes and eukaryotes (Fig. 5.7). except for a few differences (Table.1) It occurs in three steps. (1) binding of tRNA to the A site of ribosome, (2) transfer of the growing polypeptide chain from tRNA in P site to tRNA at A site by peptide bond formation. (3) translocation of the ribosome along the mRNA to position the next codon in the A site. In this step, the nascent polypeptide tRNA at A site and the uncharged tRNA are translocated from A and P sites to P and E sites respectively. These three steps are repeated in a cyclic manner through of the process of elongation. The peptidyl-tRNA present in A site of ribosome is translocated to the P site, the uncharged tRNA in P site is translocated to E site, when the ribosome moves three nucleotides towards 3' end of mRNA molecule. The translocation step requires GTP and elongation factor G (Ef-G).

Polypeptide chain elongation proceeds rapidly in *E. coli*. An amino acid to the growing polypeptide is added in about 0.05 seconds. Thus the synthesis of polypeptide containing 300 amino acids, takes only about 15 seconds.

5.2.3 Termination of Translation

When the chain enters the termination codons (UAA, UAG, UGA) they are recognized by release factors (RF) (Fig. 5.8). In *E. coli*, RF-I, RF-2 recognizes termination codons. In eukaryotes, a single release factor (eRF) recognizes all three termination codons.

This reaction releases the polypeptide from tRNA mole in P site, triggers the translocation of free tRNA to the E site. Termination is completed by the release of mRNA molecule from the ribosome and the dissociation of the ribosome into two units. Thus ribosomal subunits are then ready to initiate another round of protein synthesis as described earlier.

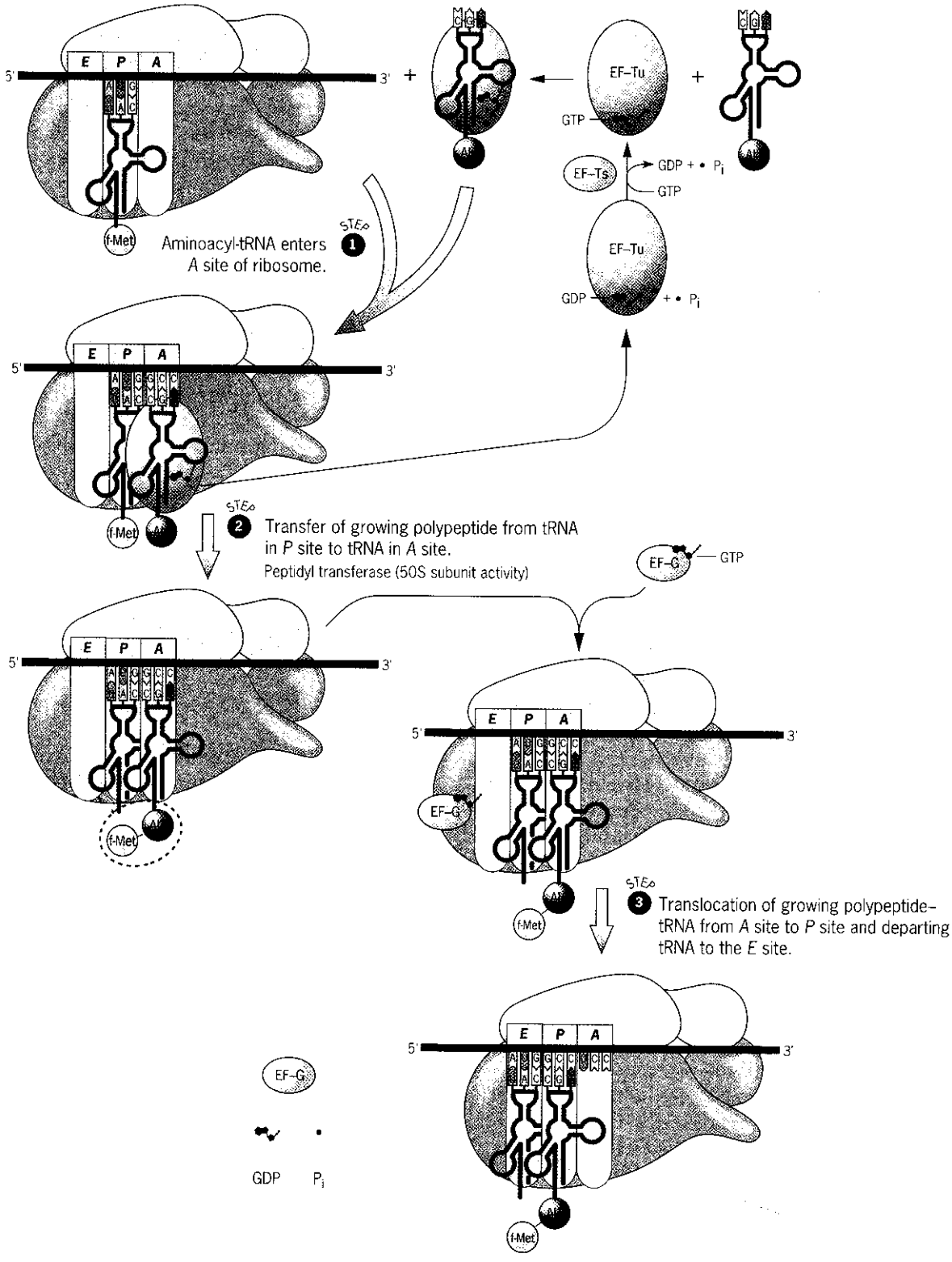


Fig. 5.7 Polypeptide chain elongation in *E. coli.*

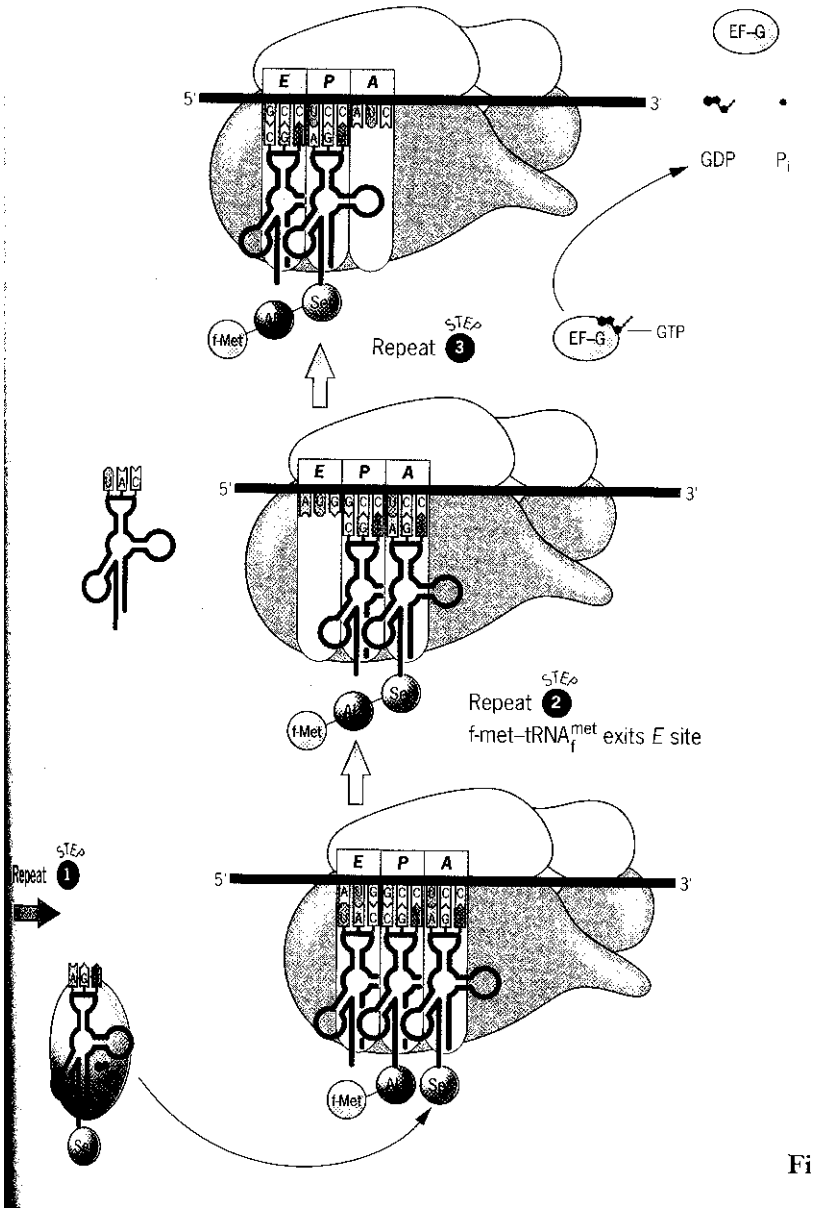


Fig. 5.7 (contd.) Polypeptide chain elongation in *E. coli*.

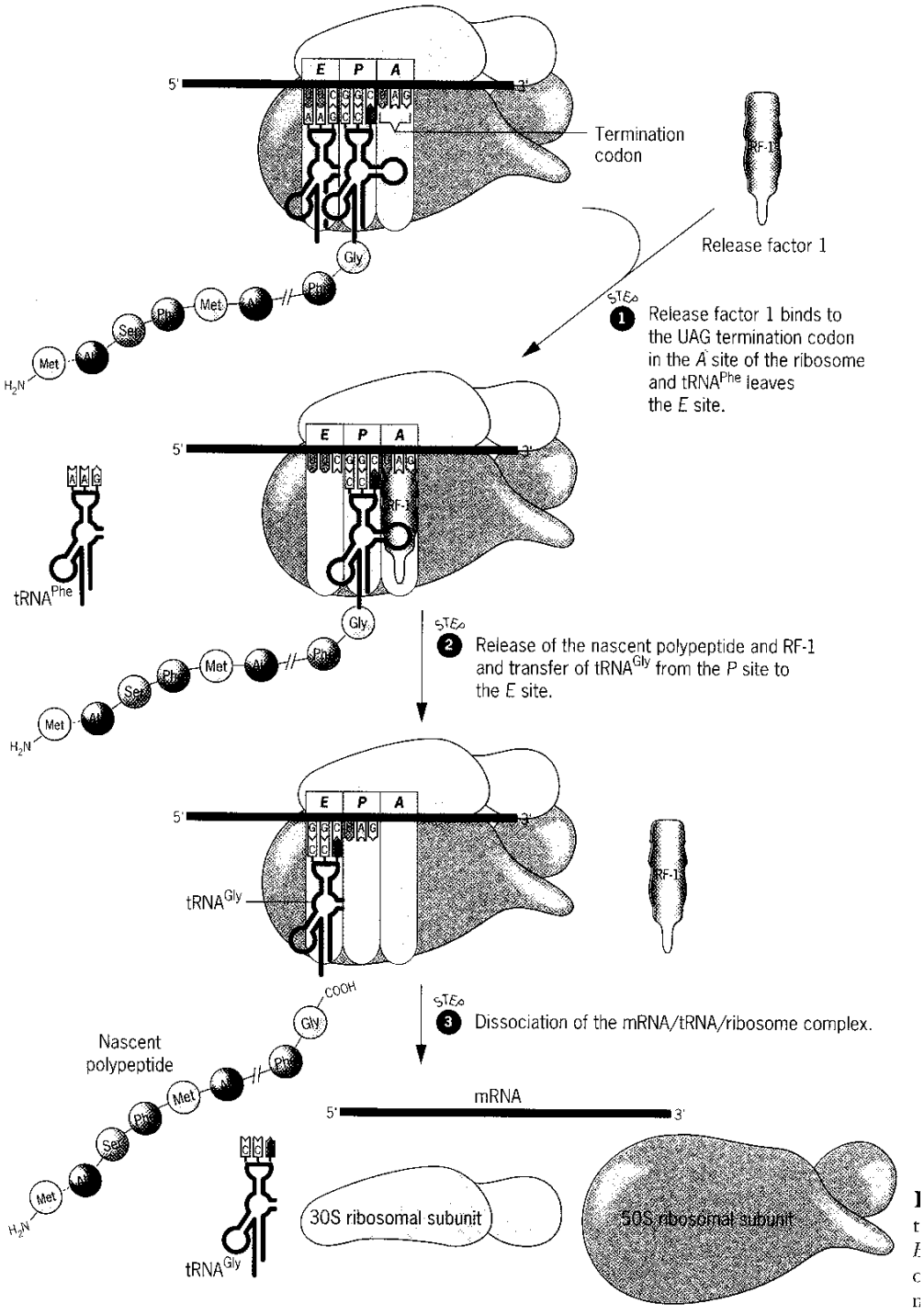


Fig. 5.8 Polypeptide chain termination in *E. coli*. The formyl group of formylmethionine is removed during translation.

5.3 Genelic code

The main features of genetic code were worked out during 1960s. Francis Crick and colleagues in 1961 published the first strong evidence in support of a triplet code through the genetic analysis of mutations by proflavin induced at rII locus of T_4 bacteriophage.

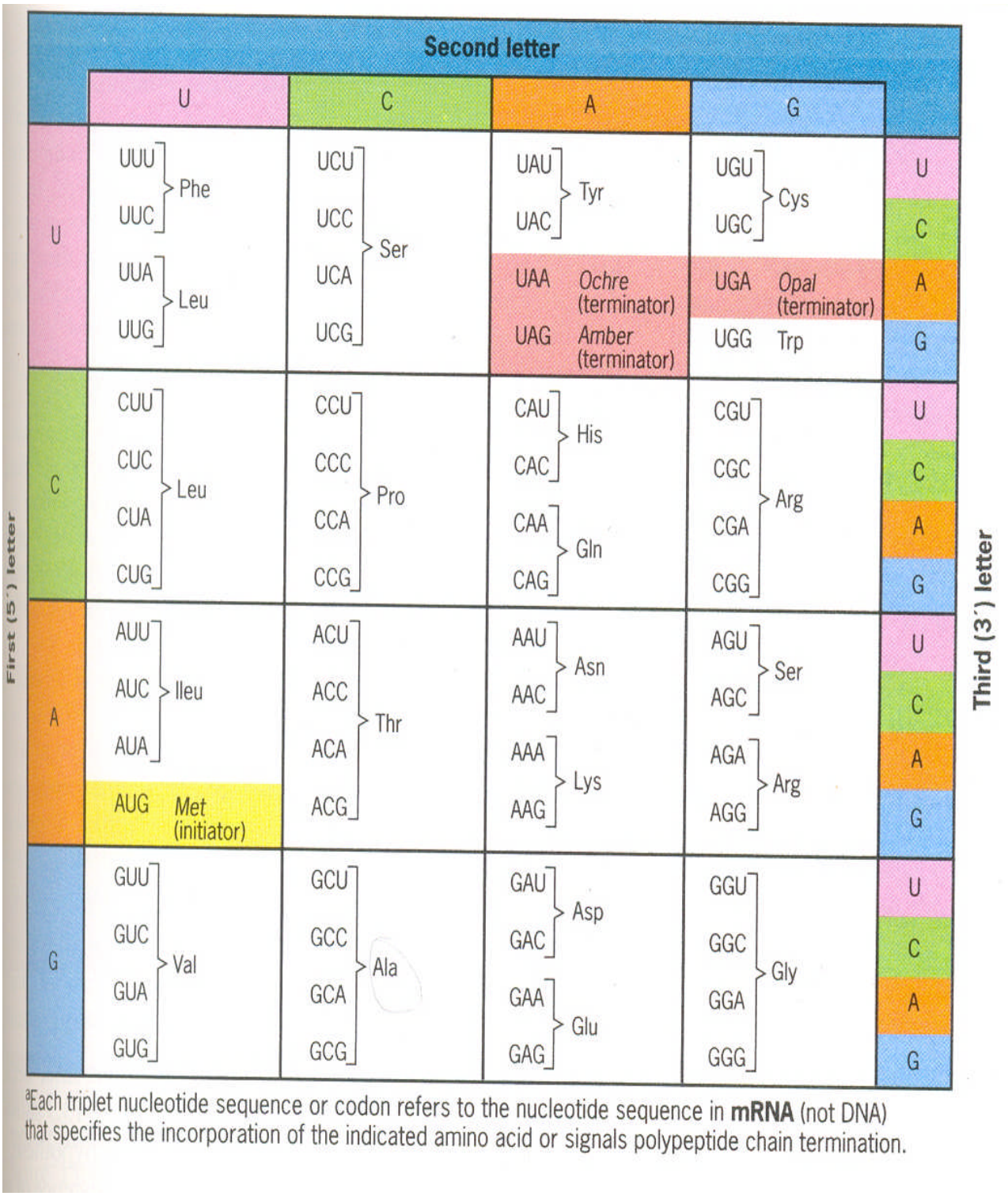
5.3.1 Experiments deciphering the genetic code

The deciphering the genetic code required scientists to obtain several answers to several questions. 1) Which codous specify each of the 20 amino acids? (2) How many of the 64 triplet codous are utilized. (3) How is the code punctuated. Do the codons have same meaning in viruses, bacteria, plants and animals?

The major break through came in 1961 when Marshall Nirenberg and Heinrich Mathei demonstrated that artificial mRNAs can be used to direct in vitro protein synthesis using ribosome, tRNAs, soluble factors. It results only one type of polypeptide by using certain mRNAs. The first code established was uuu for phenylalanize, **ccc** for proline, **AAA** for lysine, **GGG** for glycine.

In the next step, synthetic mRNAs were used in random order or in known order to probe the nature of code. From this they were able to establish the meaning which trinucleotide specified which amino acid. But it was not possible to determine the bases used in many of the code words and their specific order. Thus part of the nature of code was worked out with the synthesis of artificial mRNAs.

Additional information in the nature of the genetic code was obtained by “binding assay” method developed by Nirenberg and Leder in 1964. A given synthetic trinucleotide was mixed with ribosomes and amino acyl-t RNAs. The reaction mixture was passed over filter, which allows everything to pass through except the trinucleotide + ribosome + radioactive aminoacyl - tRNA. If the radioactivity passed through the filler, it means the radioactive amino acid was not associated with the ribosome. When radioactivity appeared on the filter, we knew that the amino acid was affiliated with the trinucleotide. Thus it is understood which amino acid is coded by the trinucleotide codon (Fig. 5.9). In this manner, in a short period of time, all the codons were deciphered (Table 2). From the table 3, salient features of genetic code can be understood.



First (5′) letter	Second letter: U	Second letter: C	Second letter: A	Second letter: G	Third (3′) letter
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA *Ochre* (terminator)	UGA *Opal* (terminator)	A
	UUG Leu	UCG Ser	UAG *Amber* (terminator)	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ileu	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ileu	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ileu	ACA Thr	AAA Lys	AGA Arg	A
	AUG *Met* (initiator)	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

[a]Each triplet nucleotide sequence or codon refers to the nucleotide sequence in **mRNA** (not DNA) that specifies the incorporation of the indicated amino acid or signals polypeptide chain termination.

Table . Genetic Code

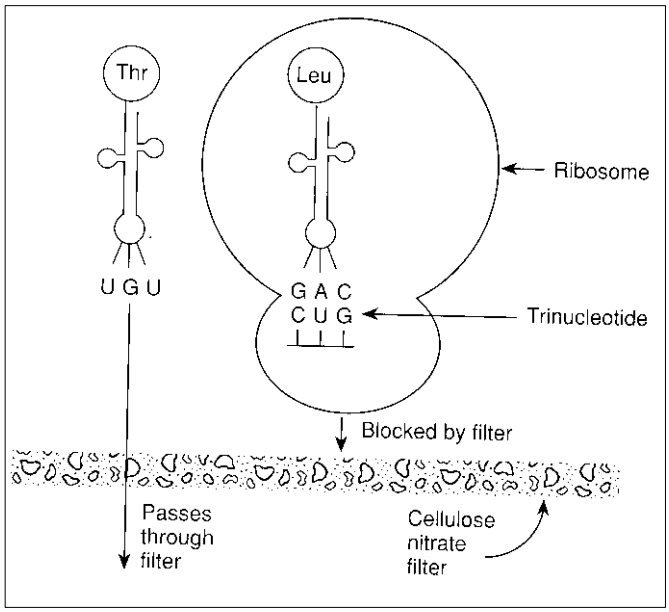


Fig. 5.9 The binding assay to determine the amino acid associated with a given trinucleotide codon. Transfer RNAs with noncomplementary codons will pass through the membrane. Transfer RNAs with anticodons complementary to the trinucleotide will bind to the ribosome and not pass through the filter. When the tRNA is charged with a radioactive amino acid, the radioactivity is trapped in the filter.

5.3.2 Features of Genetic code

Table 3 Definition of some common terms used in describing the genetic code

Terms	**Meaning**
Code letter	Nucleotide, e.g., AUGG (in mRNA) or ATGC (in DNA)
Code word, or codon Anticoden	Sequence of nucleotides specifying an amino acid, e.g., UUU=phenylalanine. Sequence of nucleotides on tRNA that complements the codon, e.g., AAA= anticodon for phenylalanine.
Genetic code or Coding dictionary Word size	A table of all the code words or codons that specify amino Acids. The number of letters in a code word, e.g., three letters in a triplet code (this is the same as **coding ratio** in a nonoverlapping code)
Non-overlapping code	When only as many amino acids are coded as there are code words in end-to-end sequence, e.g., (triplet code), UUUCCC = phenylalanine (UUU) + proline (CCC).
Overlapping code	When more amino acids are coded for more than there are code words in end-to-end sequence, e.g., UUUCCC = phenylalanine (UUU) + phenylalanine (UUC) + serine (UCC) + proline (CCC).
Degenerate code	When there is more than one codon for a particular amino acid, e.g., UUU, UUC = phenylalanine
Ambiguous code	When one codon can code for more than one amino acid, e.g., GGA =

	glycine, glutamic acid
Terms	**Meaning**
Commaless code	When there are no intermediatary nucleotides (spacers) between words, e.g., UUUCCC = two amino acids in triplet nonoverlapping code.
Reading frame	The particular nucleotide sequence that starts at a specific point and is then partitioned into codons. The reading frame may be "shifted" by removing or adding nucleotides, thereby causing a new sequence of codons to be "read".
Sense word	A codon that specifies an amino acid normally present at that position in a protein.
Missense mutation	A change in nucleotide sequence, either by deletion, insertion, or substitution, resulting in the appearance of a codon that produces a different amino acid in a particular protein, e.g. UUU (phenylalanine) $\rightarrow$ UGU (cysteine)
Nonsense mutation	A codon that does not produce an amino acid, e.g., UAG
Universality	Utilization of the same genetic code in all organisms. e.g., UUU = phenylalanine in bacteria, mouse, man and tobacco
Nearly universal Genetic code	With minor exceptions, the codes have the same meaning in all living organisms.

5.3.3 Wobble hypothesis

The first position of anticodon is not constrained as other two positions. Any base at this position can pair with any of several bases in the third position of the code. Crick characterized this ability as wobble (silent). Table 3 shows the possible pairings at 3rd codon position of mRNA. This wobble nature is of evolutionary significance to minimize the rate of mutation occurring in living organisms e.g. UUU codes phenylalanine, UUC also codes the same amino acid, phenylalanine.

Table 3 Pairing Combinations at the Third Codon Position

No.1 Base in tRNA (5' End)	**No.3 Base in mRN (3' End)**
G	U or C
C	G
A	U
U	A or G
I	A, U, or C

5.3.4 Nearly universal genetic code

Until 1979, scientists concluded that genetic code was universal. During 1979 and 1980, discrepancies were noted when mitochondrial genes deviated from the universality in the reading of the genetic code (Table 4), e.g. AUA codes amino acid Illeucine but it codes Methionine in mitochondria of yeast, CGG code Argenine but it codes Tryptophan in mitochondria of higher plants. The rule of universality of the genetic code was found to be modified in the light of findings regarding mitochondrial genes.

Table 4. Common and Alternative Meanings of Codons

Codon	**General Meaning**	**Alternative Meaning**
CUX	Leu	Thr in yeast mitochondria
AUA	Ile	Met in mitochondria of yeast, Drosophila, and vertebrates
UGA	stop	Trp in mycoplasmas and mitochondria other than higher plants
AGA/AGG	Arg	Stop in mitochondria of yeast and vertebrates Ser in mitochondria of *Drosophila*
CGG	Arg	Trp in mitochondria of higher plants
UAA/UAG	stop	Gln in ciliated protozoa

5.4 SUMMARY

The details of translation process through the steps involved in protein synthesis with the differences between prokaryotes and eukaryotes is described in this lesson. The experiments of Crick, Nirenberg and Mathaei and Nirenberg and Leder were described that have led to the elucidation of genetic code. Features of genetic code while emphasizing the wobble nature and nearly universality of genetic code and its evolutionary significance were explained.

5.5 MODEL QUESTIONS

1. Describe the process of protein synthesis while mentioning the important features of difference between prokaryotes and eukaryotes.
2. Explain the features of genetic code.
3. Write notes on:
   a) Triplet binding assay
   b) Initiation of Translation complex
   c) Wobble nature of code
   d) rRNA gene transcript.

5.6 REFERENCE BOOKS

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Prof. T.N. MARY

M.Sc. BOTANY (Final)

Paper-VII : CELL BIOLOGY AND MOLECULAR BIOLOGY

Unit-IV

Lesson 6
Gene Regulation in Prokaryotes with a brief notes on ways of Eukaryotic Gene Expression

6.0 **OBJECTIVE:**

In the earlier lessons, the process of gene expression and how the biological information contained in the DNA sequence of a gene is utilized by the cell is discussed. In this lesson, the regulation of gene expression by operon in *E. coli*, with the examples of Lactose and Tryptophan operons are described and the control levels of gene expression in eukaryotes are given here.

6.1 INTRODUCTION

The genome of a cell contains its DNA sequence having information to make many thousands of different proteins and RNA molecules. A cell typically expresses, only a fraction of its gene. Most cells continuously synthesize ribosomes and have continuous requirement for transcription of rRNA and ribosomal protein genes. Genes coding for RNA polymerase and involved in basic metabolic pathways are active in all cells through out the time. They are sometimes called **house keeping** genes. Such genes are said to be expressed constitutively and referred to as **constitutive genes**. On the other hand, many genes have a more specialized role and their biological information is needed by the cell only in certain circumstances, genes whose

expression is regulated in this manner are called **inducible genes**. This is true of genes from simplest bacteria to complex multicellular forms such as man.

Bacteria possess a number of genes coding for enzymes that enable the cell to metabolize different sugars and carbon compounds. Bacteria could continuously express all its genes, but this would waste energy, such genes are switched off when the gene products are not required. Instead, bacteria express, only those genes needed to metabolize sugars in its growth medium. Then the genes are switched on. By regulating the expression of their genes, bacteria are able to respond quickly to changes in the environment.

Although gene expression can be regulated at many levels, transcriptional regulation is the most common. In this lesson gene regulation of lactose operon and Tryptophan operon are described.

6.2 POSITIVE AND NEGATIVE CONTROL OF GENE EXPRESSION

The regulation of gene expression – induction or turning **genes on**, and repression or turning **genes off** – can be accomplished by both **positive** and **negative control** mechanism, positive and negative regulatory mechanisms are illustrated in Fig. 6.1. In positive control mechanism, the product of a regulator gene, an activator is required to turn on the expression of the structural genes. In negative control mechanisms, the product of a regulator gene, a repressor is necessary to turn off the expression of the structural genes.

In bacteria genes with related functions frequently occur in coordinately regulated units called, operons. Each operon contains a set of contiguous structural genes, a promoter and an operator.

6.3 OPERON

The operon model was developed by Francis Jacob and Jacques Monod in 1961 to explain the regulation of genes for lactose utilization in *E. coli*. The transcription of a set of structural genes is regulated by controlling elements.

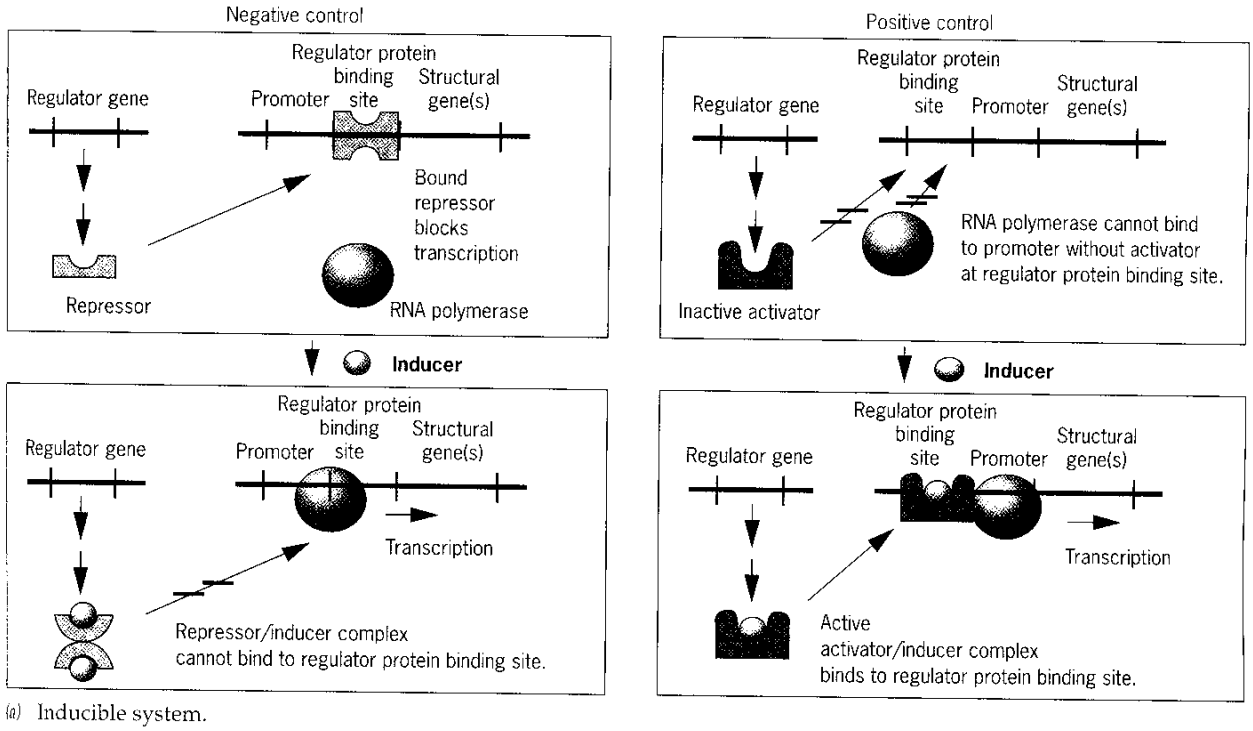
Operon - A cluster of genes that are transcribed into a single RNA molecule.

Promoter - The DNA binding site for RNA polymerase

Regulator - A gene involved in the control of the production of another gene products.

Operator - The DNA binding site, to which the repressor must attach to prevent transcription of gene or operon.

Inducer - A molecule that binds to a repressor and prevents the repressor from attaching to its operator.



(a) Inducible system.

Fig. 6.1 Negative and Positive control of inducible gene system

Corepressor - A molecule that must bind to repressor before repressor can attach to its operator.

The complete continguous unit including the structured genes, the operator and the promoter is called an operon.

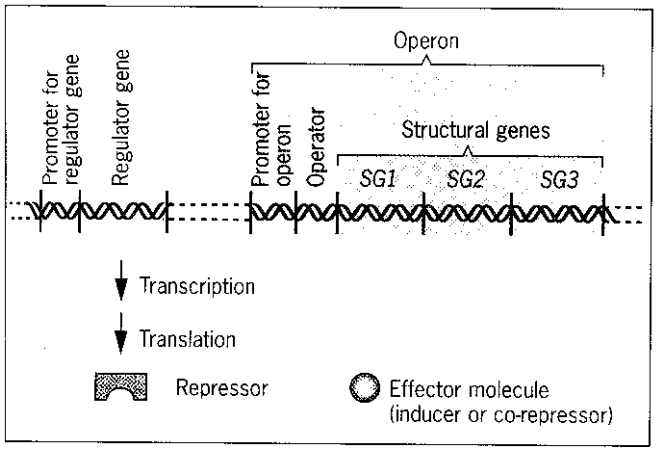
6.4 LACTOSE OPERON

Lactose is (milk sugar) used by *E. coli* for energy as a carbon source. The enzyme β-galactosidase converts lactose to allolactose. When the synthesis of the above enzyme is encoded by lacZ, the production of two additional enzymes **β-galactoside permease** (lac Y gene) and **β-galactoside**. **Acetyl transferase** (**lac** A gene) is also induced. The permease is involved in concentrating lactose in the cell. The **transferase** protect the cell from build up of toxic products.

The transcription of a set of continuous structural genes is regulated by repressor and operator gene. Transcription is initiated at promoters located upstream from the coding region of the structural genes. When repressor binds to the operator, it prevents RNA polymerase from transcribing the structural genes in an operon. Operator regions are usually located between promoters and the structural genes they regulate.

Lac operon contains promoter (P), an operator (O) and three structural genes **lac** Z, **lac** Y and **lac** A encoding the enzymes β-galactosidase, β-galactoside permease, β-galactoside transacytylase respectively. (Fig. 6.2).

In the absence of inducer, the repressor binds to the **lac** operator, which in turn prevents RNA polymerase from catalyzing the transcription of the three structural genes (Fig. 6.3). The inducer of the operon, allolactose, derived from lactose is bound by the repressor, causing the release of the repressor from the operator. In this way allolactose induces the transcription of **lac** Z, **lac** Y and **lac** A structural genes.



(a) The operon: components.

Fig. 6.2 Regulation of gene expression by the operon mechanism.

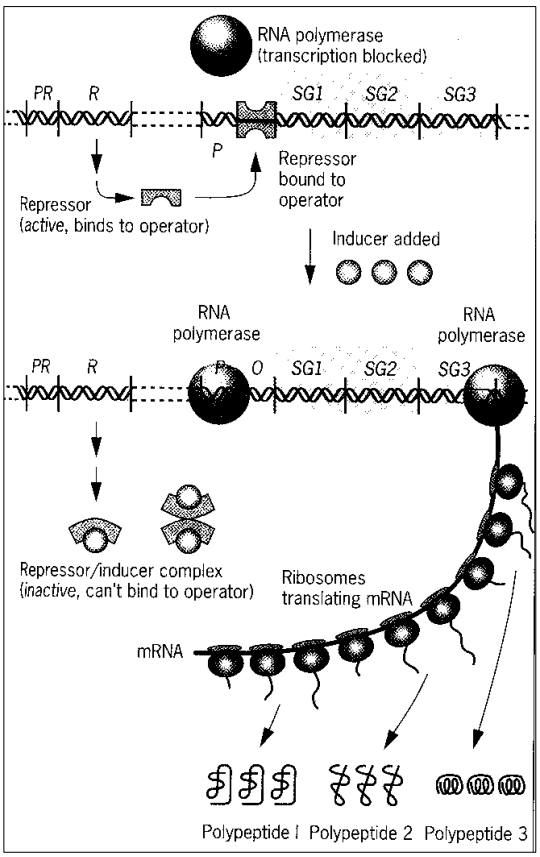


Fig. 6.3 The operon: induction (Repressible operon)

Mutations at promoter do not change the inducibility of the **lac** operon. They modify the levels of gene expression in the induced and uninduced state by changing the initiation of **lac** operon transcription – efficiency of RNA polymerase binding.

Lac promoter (Fig. 6.4) contains two separate components: (1) RNA polymerase binding site (2) a binding site for another protein called catabolite activator protein (CAP) that prevents the **lac** operon from being induced in the presence of glucose.

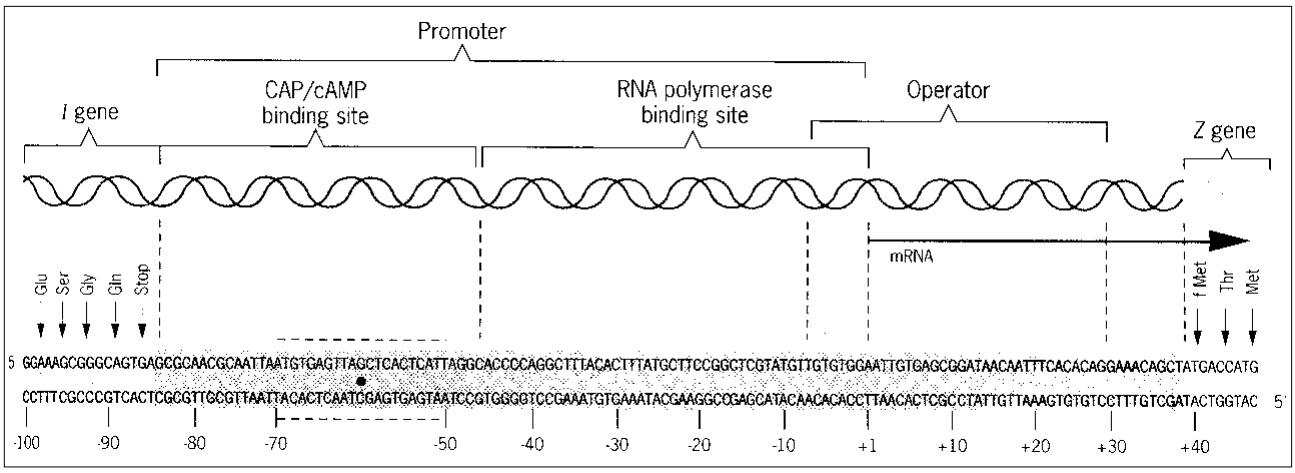


Fig. 6.4 Organization of the promoter-operator region of the **lac** operon. The promoter consists of two components: (1) the site that binds the CAP/cAMP complex and (2) the RNA polymerase binding site. The adjacent segments of the **lacI** (repressor) and lacZ (β-galactosidase) structural genes are also shown. The horizontal line labeled mRNA shows the position at which transcription of the operon begins (the 5'-end of the **lac** mRNA). The numbers at the bottom give distances in nucleotide pairs from the site of transcript initiation (position +1). The dot between the two nucleotide strands indicates the centre of symmetry of an imperfect palindrome.

6.4.1 Catabolite Repression

The presence of glucose prevent the induction of the **lac** operon, as well as other operon controlling enzymes involved in carbohydrate catabolism, glucose is catabolized in preference to other sugars. This phenomenon is called catabolite repression (glucose effect).

The phenomenon of catabolite repression of the **lac** operon and several other operons is mediated by a regulatory protein called CAP and a small effector molecule called cyclic AMP (adenosine-3',5'-monophosphate cAMP). cAMP acts as the effector molecule, determining the effect of CAP on **lac** operon transcription (Fig. 6.5).

In the absence of glucose, cAMP combines with CAP and CAP-cAMP complex binds to a distal part of the promoter of operons with CAP site. This binding apparently enhances the affinity of RNA polymerase for the promoter, because without the binding of CAP-cAMP complex to the promoter, the transcription rate is very low. The binding of CAP-cAMP to CAP sites causes the DNA to bend by more than 90^o (Fig. 6.6). This bending causes for enhanced transcription, making the DNA more available to RNA polymerase.

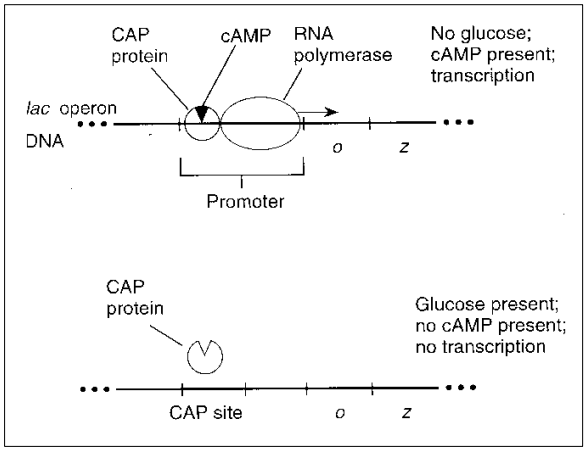


Fig. 6.5. Catabolite represssion. When cAMP is present in the cell (no glucose is present), it binds with CAP protein and together they bind to the CAP site in various sugar-metabolizing operons, such as the **lac** operon shown here. The CAP-cAMP complex enhances the transcription of theoperon. When glucose is present, it inhibits the formation of cAmP. Thus no CAP-cAMP complex is formed, and therefore transcription of the same operons is not enhanced.

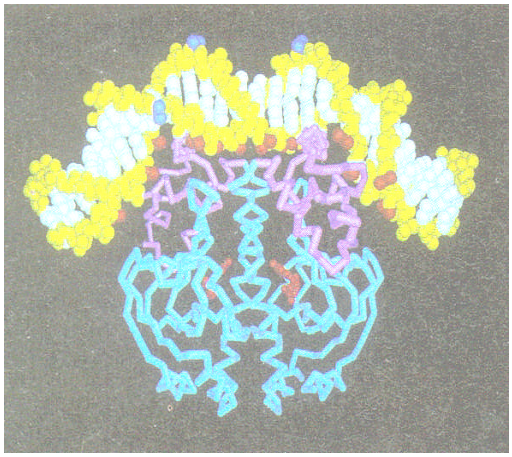


Fig. 6.6 CAP-DNA interaction. Model of cap protein and DNA. The cAMP-binding domain is blue, DNA-binding domain is purple, and the cAMP are red.

Catabolite repression is an example of positive regulation. Binding of the CAP-cAMP complex at CAP site enhances the transcription rate. Thus **lac** operon is both positively and negatively regualted.

6.5 TRYPTOPHAN OPERON (REPRESSIBLE SYSTEM)

6.5.1 Repression

The **trp** operon of *E. coli* is a negative repressible operon. The organization of Tryptophan operon and the pathway of biosynthesis of tryptophan are shown in Fig. 6.7).

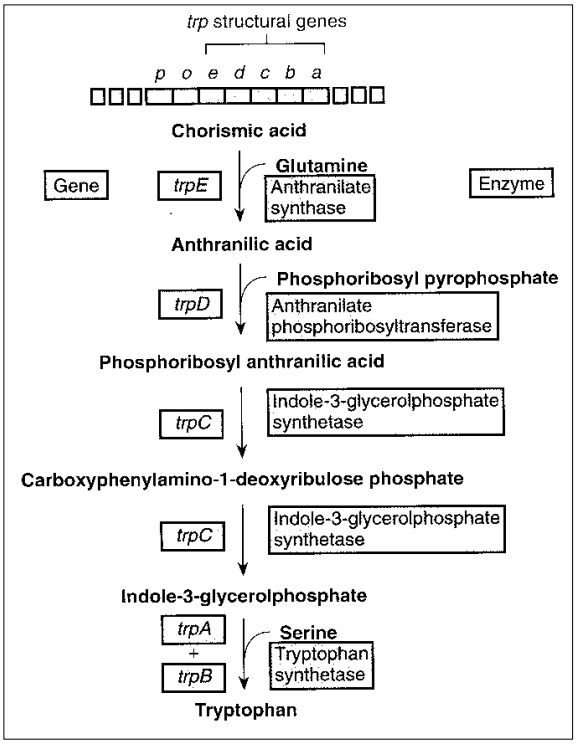


Fig. 6.7 Genes of the tryptophan operon in *E. coli*. The enzymes the produce control the conversion of chorismic acid to tryptophan. The symbol o on the chromosome refers to the trp operator, which has its own repressor, the product of the trpR gene. →

In the absence of tryptophan (co-repressor), RNA polymerase binds to the promoter region and transcribes the structural genes of the operon. In the presence of tryptophan, the co-repressor/ repressor complex binds to the operator region and prevents RNA polymerase from initiating transcription of the genes in the operon. This is operator control mechanism (Fig. 6.8). This regulation is modified, by the existence of second mechanism for regulating repressible operons by attenuator control.

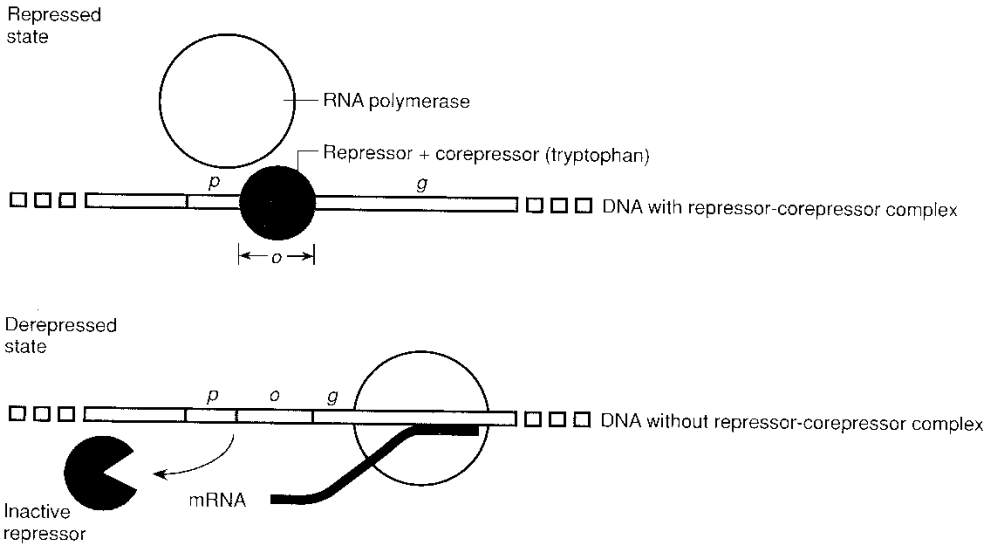


Fig. 6.8 The repressor-corepressor complex binds at the operator and prevents the transcription of the *trp* operon in *E. coli*. Without the corepressor, the repressor cannot bind and therefore transcription is not prevented from occurring.

6.5.2 Attenuation

The second level of regulation of try operon is called attenuation and the sequence that controls this phenomenon is called the attenuator. Attenuator region (Fig. 6.9) has a nucleotide – pair sequence identical to the transcription – termination signals.

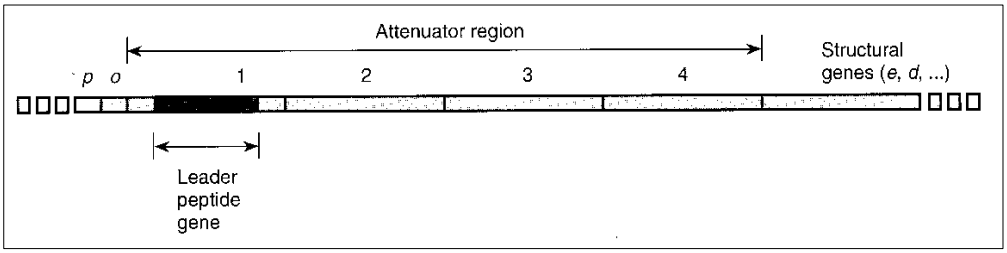


Fig. 6.9 Attenuator region of the *trp* operon, which contains the leader peptide gene. This region is transcribed into the leader transcript.

The attenuator region between operator and structural genes consists of leader transcript having four subregions of mRNA 1-2 and 3-4.

The gene for leader peptide codes for fourteen amino acids, of which two adjacent codons code for tryptophan (Fig. 6.10).

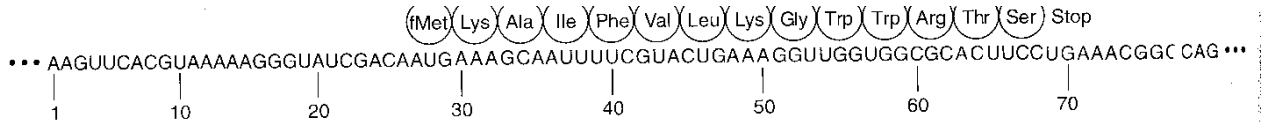


Fig. 6.10 Base sequence of the *trp* leader peptide gene and the amino acids coded for by these nucleotides. Note the presence of adjacent tryptophan codons.

6.5.3 Excess Tryptophan

Depending on the levels of amino acids in the cell, three outcomes of this translation process can take place. If the concentration of tryptophan in the cell is high (Fig. 6.11), the ribosome will over lap regions 1 and 2 of the transcript and allow 3-4 stem loop to form. This stem-loop structure is referred to as the terminator or attenuator stem and cause the transcription to be terminated.

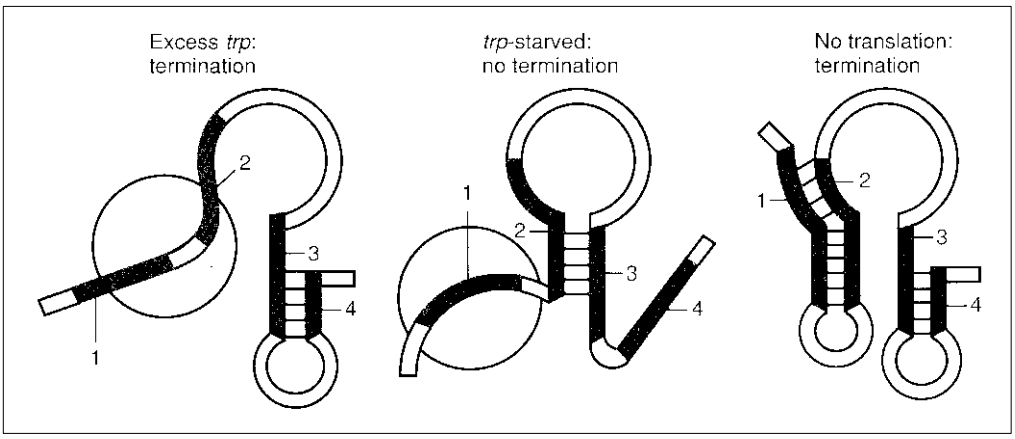


Fig. 6.11 Model for attenuation in the *E. coli trp* operon. The circle represents the ribosome.

6.5.4 Tryptophan starvation

If there is lowered quantity of tryptophanyl - tRNA, the ribosome will have to wait at the first tryptophan codon (Fig. 6.11). The stalled ribosome will permit 2-3 stem loop to form,

preventing the formation of 3-4 stem loop. Then the operon is translated, raising the level of tryptophan in the cell. The 2-3 stem loop structure is referred to as the preemptor stem.

Regulation of transcription by attenuation is not unique to the **try** operon. Five other operons (thr, ilu, leu, phe and his) are known to be regulated by attenuation. Although minor details vary from operon to operon, the main features of attenuation are the same for all the above operons.

6.6 REGULATION OF GENE ACTIVITY IN EUKARYOTES

More than two hundred cell types have been identified in the human body, including those for muscle, liver, nerve, lung, epithelium, bone marrow (B), and thymus (T) lymphocytes. These cell types differ dramatically in both morphology and function. However, with some exceptions, the genetic information of the various cells within a given organism is virtually the same. These different cell types seem to arise mainly from the differential expression of an identical genome, which is due to the expression of a characteristic subset of mRNAs and proteins in any given type of cell. The majority of the proteins and mRNAs however, are produced in all cell types, and their levels do not differ by more than a few fold from one cell to another (i.e., they are responsible for so-called housekeeping functions). Eukaryotes have evolved complex regulatory mechanisms to express a specific gene in one cell type, but not in another without changing the sequence of the gene itself. In principle, gene expression can be regulated at each step in the circular synthesis/modification pathway from DNA to RNA to protein to DNA (Fig. 6.12). Potentical control levels include:

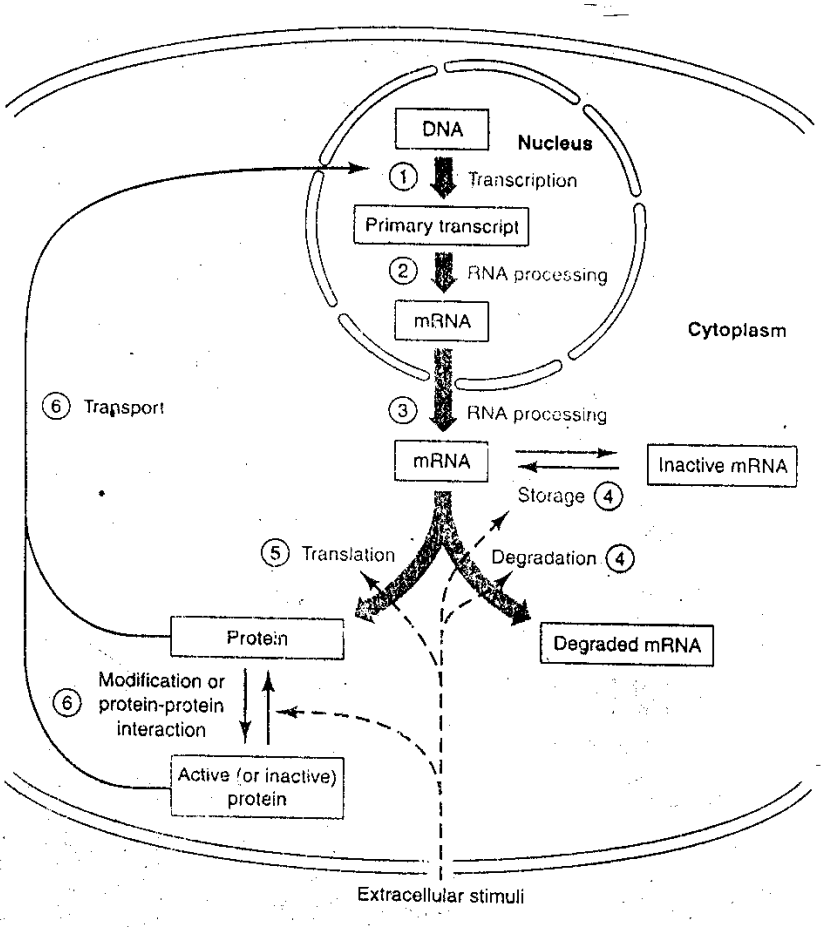


Fig. 6.12 Potential control levels for the regulation of gene expression in eukaryotes. Levels 1 and 2 are localized in the nucleus and 4 and 5 in the cytoplasm; 3 and 6 indicate translocation between the two compartments. Note that with 6 only one possible route of protein translocation (from the cytoplasm to the nucleus) is indicated RNA metabolism is shown in black.

1. Transcription
2. RNA processing (splicing or other types of processing modification)
3. mRNA transport
4. mRNA degradationa nd storage
5. Translation
6. Post-translational modulation f protein activity

6.7 SUMMARY

The concept of operon, the regulation gene expression, in terms of an inducible gene and repressible genes are explained. Positive and negative control mechanisms are illustrated. Lactose utilization in *E. coli* by inducible genes regulation is described. The phenomenon of catabolite regression mediated by regulatory protein (CAP) and a small effector molecule cyclic AMP and their significance in glucose metabolism are briefed in this lesson. The regulation of Tryptophan operon in *E. coli* at the level of repression and attenuation are discussed.

6.8 MODEL QUESTIONS

1. Discuss gene regulation with regard to lactose operon.
2. Describe catabolite repression.
3. Explain the attenuator control of gene regulation in Tryptophan operon.
4. Write notes on:
   a) Leader peptide
   b) Repressible gene expression
   c) Induced genes
   d) Operon model

6.9 REFERENCE BOOKS

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