UNIT - I

1) Introduction of Genetic Engineering

2) Recombinant DNA Technology

i) Tools: -

A) Enzymes: -

a) Lysing
b) Ligases
c) Nucleases (Exonucleases, Endonucleases, Restriction Endonucleases)
d) Synthetases (DNA polymerase, Reverse transcriptase)

B) Vectors: - Cloning vectors
(Plasmid -psBR322, Bacteriophage-Lambda phage,
Virus- SV40, Cosmid vectors)

3) Techniques: -

i) Gel-Electrophoresis
ii) PCR (Polymerase Chain Reaction)
iii) Southern, Northern and Western Blotting.

1. Introduction of Genetic Engineering

Genetic engineers have developed genetic recombination techniques to manipulate gene sequences in plants, animals and other organisms to express specific traits. Applications for genetic engineering are increasing as engineers and scientists work together to identify the locations and functions of specific genes in the DNA sequence of various organisms. Once each gene is classified, engineers develop ways to alter them to create organisms that provide benefits such as cows that produce larger volumes of meat, fuel- and plastics- generating bacteria, and pest-resistant crops.

Genetic engineering is the process of transferring specific genes from the chromosome of one organism and transplanting them into the chromosome of another organism in such a way that they become a reproductive part of the new organism. The process that produces the resulting recombinant DNA involves four steps:

1. The desired DNA is cleaved from the donating chromosome by the action of *restriction enzymes*, which recognize and cut specific nucleotide segments, leaving a "sticky end" on both ends. The restriction enzymes also splice the receiving chromosome in a complementary location, again leaving "sticky ends" to receive the desired DNA.
2. The desired DNA fragment is inserted into a vector, usually a plasmid, for transfer to the receiving chromosome. Plasmids are an ideal vector because they replicate easily inside host bacteria and readily accept and transfer new genes. Plasmids are circular DNA molecules found in the cytoplasm of bacteria that bond with the desired DNA fragment with the help of the joining enzyme, *DNA ligase*, to create the resulting *recombinant DNA*.
3. When the host cell reproduces, the plasmids inside also reproduce, making multiple clones of their DNA. Because the plasmid DNA contains the desired as well as unwanted DNA clones, the entire product is referred to as a *gene library*. The desired gene is similar to one book in that library.
4. To recover the desired DNA, the current technology is to screen unwanted cells from the mixture and then use gel electrophoresis to separate the remaining genes by movement on an electric grid. Gel electrophoresis uses a positively charged grid to attract the negatively charged DNA fragments, thereby separating them by size, because the smaller ones will migrate the most. Radioactive or fluorescent probes are added, which attract and bind with the desired DNA to produce visible bands. Once isolated, the DNA is available for commercial use.

1.1 Historical Developments

The term *genetic engineering* initially referred to various techniques used for the modification or manipulation of organisms through the processes of heredity and reproduction. As such, the term embraced both artificial selection and all

the interventions of biomedical techniques, among them artificial insemination, in vitro fertilization (e.g., “test-tube” babies), cloning, and gene manipulation. In the latter part of the 20th century, however, the term came to refer more specifically to methods of recombinant DNA technology (or gene cloning), in which DNA molecules from two or more sources are combined either within cells or in vitro and are then inserted into host organisms in which they are able to propagate.

The possibility for recombinant DNA technology emerged with the discovery of restriction enzymes in 1968 by Swiss microbiologist Werner Arber. The following year American microbiologist Hamilton O. Smith purified so-called type II restriction enzymes, which were found to be essential to genetic engineering for their ability to cleave a specific site within the DNA (as opposed to type I restriction enzymes, which cleave DNA at random sites). Drawing on Smith’s work, American molecular biologist Daniel Nathans helped advance the technique of DNA recombination in 1970–71 and demonstrated that type II enzymes could be useful in genetic studies. Genetic engineering based on recombination was pioneered in 1973 by American biochemists Stanley N. Cohen and Herbert W. Boyer, who created an interesting model for screening the host cells to finds the desired DNA fragment. In their experiment, they inserted the desired DNA and a DNA segment that made the host bacteria resistant to a particular antibiotic, tetracycline. When the antibiotic was applied to the general population, only those bacteria that had received the plasmid survived—so they knew their desired DNA fragment was located in the surviving bacteria.

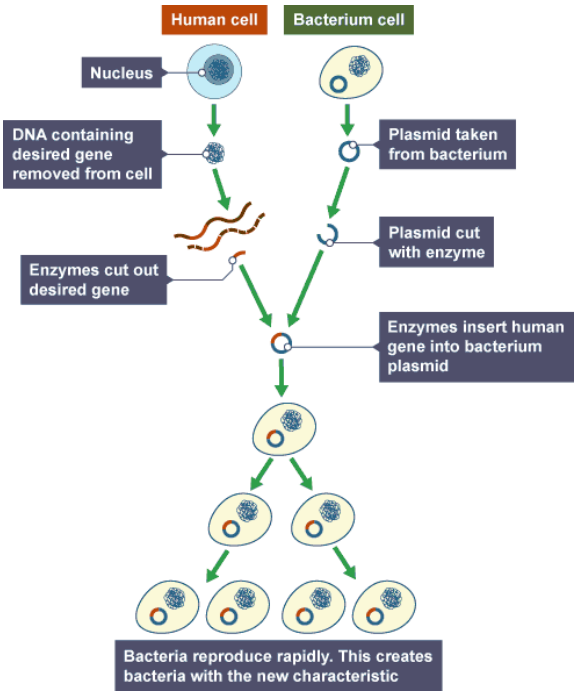


Fig. 1.1

2. Recombinant DNA Technology

Recombinant DNA technology**,** joining together of DNA molecules from two different species that are inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, and industry. Since the focus of all genetics is the gene, the fundamental goal of laboratory geneticists is to isolate, characterize, and manipulate genes. Although it is relatively easy to isolate a sample of DNA from a collection of cells, finding a specific gene within this DNA sample can be compared to finding a needle in a haystack. Consider the fact that each human cell contains approximately 2 metres (6 feet) of DNA. Therefore, a small tissue sample will contain many kilometres of DNA. However, recombinant DNA technology has made it possible to isolate one gene or any other segment of DNA, enabling researchers to determine its nucleotide sequence, study its transcripts, mutate it in highly specific ways, and reinsert the modified sequence into a living organism.

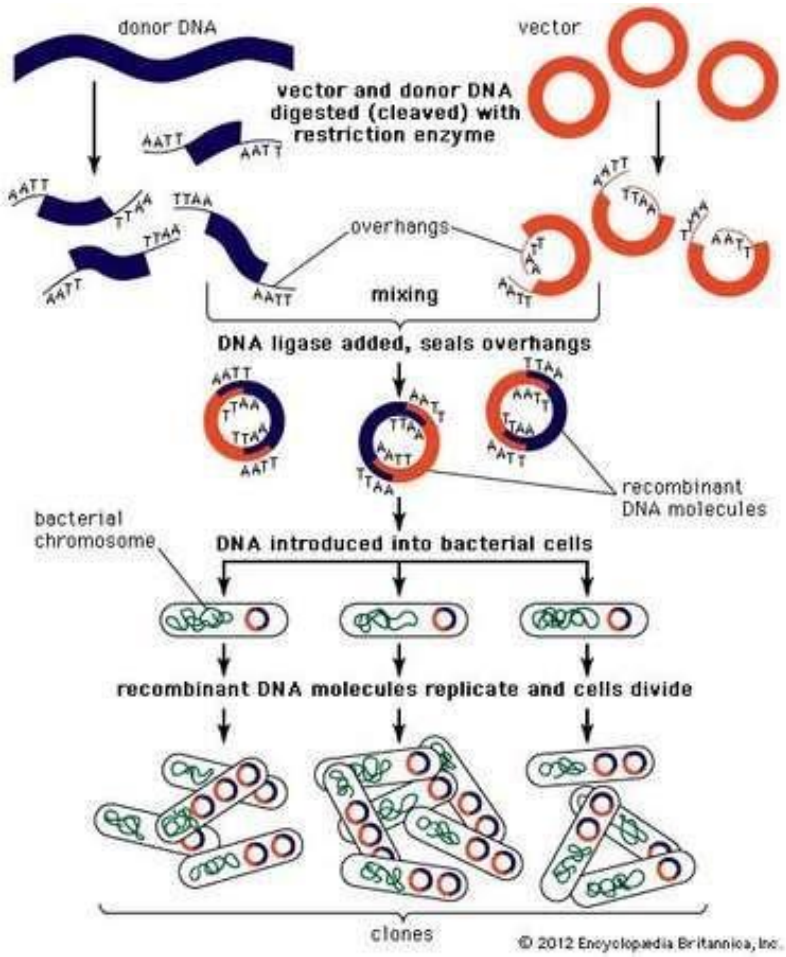


Fig. 1.2

As well as having what might be termed a good 'infrastructure' (a good laboratory setup, with access to various essential items of equipment), the genetic engineer needs to be able to cut and join DNA from different sources. This is the essence of creating recombinant DNA in the test tube. In addition, certain modifications to the DNA may have to be carried out during the various steps required to produce, clone, and identify recombinant DNA molecules. The tools that enable these manipulations to be performed are enzymes, which

are purified from a wide range of organisms and can be bought from various suppliers. In this chapter we will have a look at some of the important classes of enzymes that make up the genetic engineer's toolkit.

Tools for the GE

A) Enzymes use in Genetics Engineering

a. Ligases:

In molecular biology and genetics engineering, ligase enzymes are called molecular glue or molecular paste. The action of ligase is opposite to that of RE restriction endonuclease.

In the cell the function of DNA ligase is to repair single-stranded breaks ("discontinuities") that arise in double-stranded DNA molecules during DNA replication. DNA ligases from most organisms can also join together two individual fragments of double-stranded DNA. Ligase catalyzes the ligation or joining of nucleic acid fragments by forming a phosphodiester bond between the directly adjacent 3'-OH and 5'-P termini.

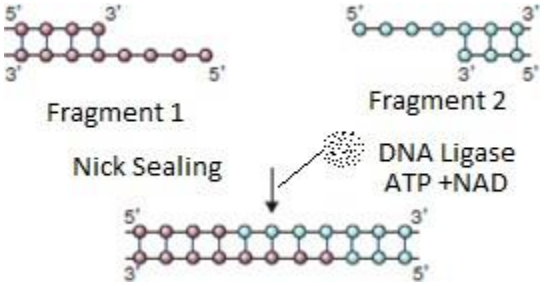


Fig. 1.3 Ligation takes place between 5'-P and 3'-OH termini

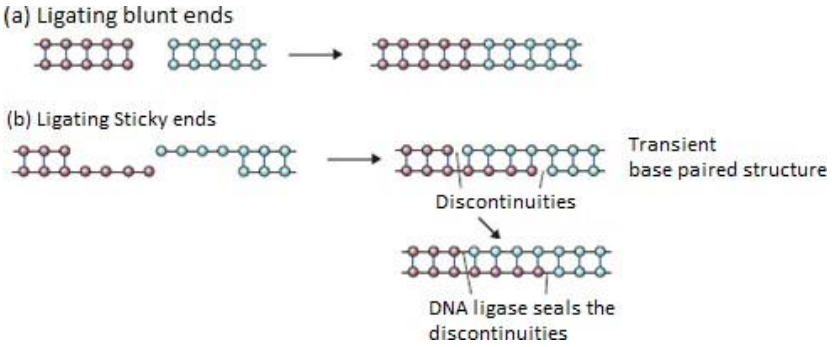


Fig 1. 4. The Different joining catalyzed by DNA ligase (a) Ligation of blunt ended molecule (b) ligation of sticky -ended molecule

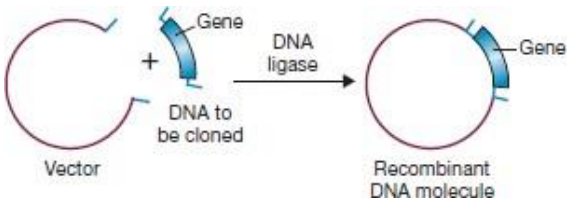


Fig 1.5. Ligation: the final step in construction of a RDNA molecule

The ligation reaction in Figure 4.20a shows two blunt-ended fragments being joined together. Although this reaction can be carried out in the test tube, it is not very efficient. This is because the ligase is unable to "catch hold" of the molecule to be ligated, and has to wait for chance associations to bring the ends together. If possible, blunt end ligation should be performed at high DNA concentrations, to increase the chances of the ends of the molecules coming together in the correct way.

In contrast, ligation of complementary sticky ends is much more efficient. This is because compatible sticky ends can base pair with one another by hydrogen bonding (Figure 4.20b), forming a relatively stable structure for the enzyme to work on. If the phosphodiester bonds are not synthesized fairly quickly then the sticky ends fall apart again. These transient, base-paired structures do, however, increase the efficiency of ligation by increasing the length of time the ends are in contact with one another.

Types of ligase: there are three types of Ligase

- *E.coli*. ligase 75kD which catalyzes the cohesive and blunt-end ligation in the presence of NAD as a coenzyme.
- Bacteriophage T4 ligase of 68 kD which catalyzes the ligation of cohesive ends, blunt end, DNA-RNA hybrid, and RNA-RNA hybrids, in the presence of ATP as coenzyme.
- *Taq* DNA ligase obtained by cloning the *Taq* gene into *E. coli.*

Uses:

- To complete lagging strand synthesis during replication
- To ligate vectors and foreign gene during e-DNA technology.
- To ligate linker or adaptors at the blunt ends of DNA fragments
- To seal nicks in the second strand during cDNA synthesis.
- To amplify DNAs.
- To detect point mutation in DNAs by ligase chain reaction or ligase amplification.
- In DNA repair.

c) Nucleases

Nucleases are enzymes that hydrolyze nucleic by breaking the phosphodiester bond that holds the nucleotides together. Nucleases are most important classes of enzymes involved in molecular biology and genetic engineering. Nucleases are usually further divided into endonucleases and exonucleases. Restriction enzymes are good examples of endonucleases, which cut within a DNA strand. A second group of nucleases, which degrade DNA from the termini of the molecule, are known as exonucleases.

Exonucleases :

Exonucleases are enzymes that work by cleaving nucleotides one at a time from the end (exo) of a polynucleotide chain. A hydrolyzing reaction that breaks phosphodiester bonds at either the 3' or the 5' end occurs. Its close relative is the endonuclease, which cleaves phosphodiester bonds in the middle (endo) of a polynucleotide chain. Eukaryotes and prokaryotes have three types of exonucleases involved in the normal turnover of mRNA: 5' to 3' exonuclease, which is a dependent decapping protein; 3' to 5' exonuclease, an independent protein; and poly(A)-specific 3' to 5' exonuclease.

In 1971, Lehman IR discovered exonuclease I in *E. coli*. Since that time, there have been numerous discoveries including: exonuclease, II, III, IV, V, VI, VII, and VIII. Each type of exonuclease has a specific type of function or requirement.

Exonuclease I breaks apart single-stranded DNA in a 3' → 5' direction, releasing deoxyribonucleoside 5'-monophosphates one after another. It does not cleave DNA strands without **terminal 3'-OH groups** because they are blocked by phosphoryl or acetyl groups.

Exonuclease II is associated with DNA polymerase I, which contains a 5' exonuclease that clips off the RNA primer contained immediately upstream from the site of DNA synthesis in a 5' → 3' manner.

Exonuclease III has four catalytic activities:

- 3' to 5' exodeoxyribonuclease activity, which is specific for double-stranded DNA
- RNase activity
- 3' phosphatase activity
- AP endonuclease activity (later found to be called endonuclease II).

Exonuclease IV adds a water molecule, so it can break the bond of an oligonucleotide to nucleoside 5' monophosphate. This exonuclease requires **Mg 2+** in order to function and works at higher temperatures than exonuclease I.

Exonuclease V is a 3' to 5' hydrolyzing enzyme that catalyzes linear double-stranded DNA and single-stranded DNA, which requires **Ca2+**. This enzyme is extremely important in the process of homologous recombination.

Exonuclease VIII is 5' to 3' dimeric protein that does not require ATP or any gaps or nicks in the strand, but requires a **free 5' OH group** to carry out its function.

Endonucleases:

Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain. Some, such as Deoxyribonuclease I, cut DNA relatively nonspecifically (without regard to sequence), while many, typically called restriction endonuclease or restriction enzymes, cleave only at very specific nucleotide sequences.

Restriction enzymes are endonucleases from eubacteria and archaea that recognize a specific DNA sequence. The nucleotide sequence recognized for cleavage by a restriction enzyme is called the restriction site. Typically, a restriction site will be a palindromic sequence of about four to six nucleotides long. Most restriction

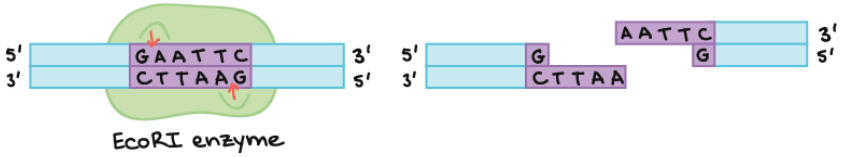
endonucleases cleave the DNA strand unevenly, leaving complementary single-stranded ends. These ends can reconnect through hybridization and are termed "sticky ends". Once paired, the phosphodiester bonds of the fragments can be joined by DNA ligase. There are hundreds of restriction endonucleases known, each attacking a different restriction site. The DNA fragments cleaved by the same endonuclease can be joined together regardless of the origin of the DNA. Such DNA is called recombinant DNA; DNA formed by the joining of genes into new combinations. *Restriction endonucleases* (restriction enzymes) are divided into three categories, Type I, Type II, and Type III, according to their mechanism of action. These enzymes are often used in genetic engineering to make recombinant DNA for introduction into bacterial, plant, or animal cells, as well as in synthetic biology.

Restriction Endonucleases

Restriction enzymes are the scissors of molecular genetics. Restriction enzymes (RE) are endonucleases that will recognize specific nucleotide sequences in the DNA and break the DNA chain at those points. A variety of RE have been isolated and are commercially available. Most cut at specific palindromic sites in the DNA (sequence that is the same on both antiparallel DNA strands). These cuts can be a staggered which generate “sticky or overhanging ends” or a blunt which generate flush ends.

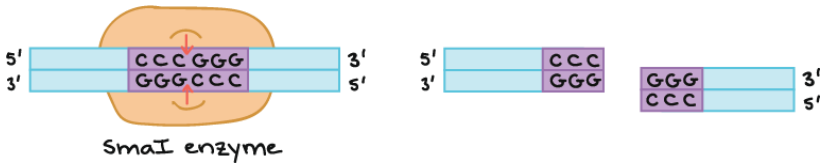
As an example of how a restriction enzyme recognizes and cuts at a DNA sequence, let's consider *Eco*RI, a common restriction enzyme used in labs. *Eco*RI cuts at the following site:

When EcoRI recognizes and cuts this site, it always does so in a very specific pattern that produces ends with single-stranded DNA “overhangs”:



If another piece of DNA has matching overhangs (for instance, because it has also been cut by EcoRI), the overhangs can stick together by complementary base pairing. For this reason, enzymes that leave single-stranded overhangs are said to produce **sticky ends**. Sticky ends are helpful in cloning because they hold two pieces of DNA together so they can be linked by DNA ligase.

Not all restriction enzymes produce sticky ends. Some are “blunt cutters,” which cut straight down the middle of a target sequence and leave no overhang. The restriction enzyme *Sma*I is an example of a blunt cutter:



Blunt-ended fragments can be joined to each other by DNA ligase. However, blunt-ended fragments are harder to ligate together (the ligation reaction is less efficient and more likely to fail) because there are no single-stranded overhangs to hold the DNA molecules in position.

Restriction enzymes are of three types (I, II, or III). Most of the enzymes commonly used today are type II enzymes, which have the simplest mode of action.

Table Recognition sequences and cutting sites for some restriction endonucleases

Enzyme	Recognition sequence	Cutting sites	Ends
*Bam*HI	5’-GGATCC-3’	G↓GAT C C C C T A G↑G	5’
*Eco*RI	5’-GAATTC-3’	G↓A AT T C C T T A A↑G	5’
*Hae*III	5’-GGCC-3’	GG↓C C C C↑GG	Blunt
*Hpa*I	5’-GTTAAC-3’	G T T↓A A C C A A↑T T G	Blunt
*Pst*I	5’-CTGCAG-3’	C T GC A↓G G↑A C G T C	3’
*Sau*3A	5’-GATC-3’	↓G A T C C T A G↑	5’
*Sma*I	5’-CCCGGG-3’	C C C↓GG G GG G↑C C C	Blunt
*Sst*I	5’-GAGCTC-3’	G A GC T↓C C↑T CGA G	3’
*Xma*I	5’-CCCGGG-3’	C↓C C GG G G G G CC↑C	5’

d) Synthetases (DNA polymerase, Reverse transcriptase)

DNA polymerases

DNA polymerases are enzymes that synthesize DNA molecules from deoxyribonucleotides, the building blocks of DNA. These enzymes are essential to DNA replication and usually work in pairs to create two identical DNA strands from a single original DNA molecule. During this process, DNA polymerase “reads” the existing DNA strands to create two new strands that match the existing ones.

These enzymes catalyze the following chemical reaction

deoxynucleoside triphosphate + $DNA_n \rightleftharpoons$ diphosphate + DNA_{n+1}

Catalyses DNA-template-directed extension of the 3'- end of a DNA strand by one nucleotide at a time.

Every time a cell divides, DNA polymerases are required to help duplicate the cell's DNA, so that a copy of the original DNA molecule can be passed to each daughter cell. In this way, genetic information is passed down from generation to generation.

Before replication can take place, an enzyme called helicase unwinds the DNA molecule from its tightly woven form. This opens up or "unzips" the double-stranded DNA to give two single strands of DNA that can be used as templates for replication.

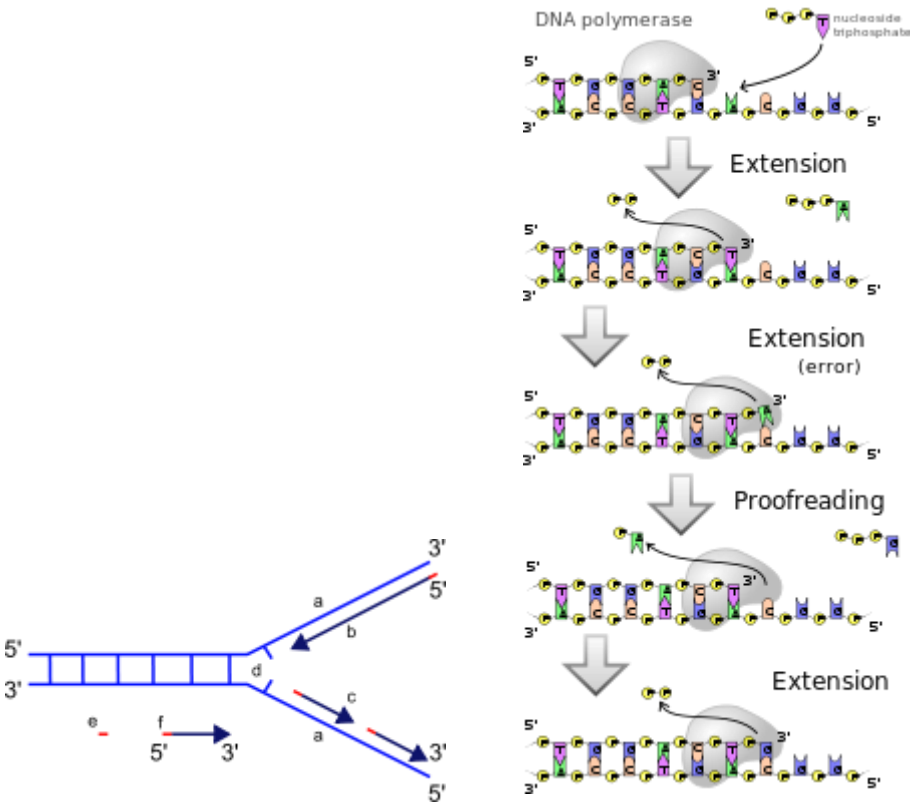


Fig. 1.6

Pol I

Prokaryotic family A polymerases include the DNA polymerase I (Pol I) enzyme, which is encoded by the *polA* gene and ubiquitous among prokaryotes. This repair polymerase is involved in excision repair with both 3'-5' and 5'-3' exonuclease activity and processing of Okazaki fragments generated during lagging strand synthesis Pol I is the most abundant polymerase, accounting for >95% of polymerase activity in *E. coli*; yet cells lacking Pol I have been found suggesting Pol I activity can be replaced by the other four polymerases. Pol I adds ~15-20 nucleotides per second, thus showing poor processivity. Instead, Pol I starts adding nucleotides at the RNA primer: template junction known as the origin of replication (ori). Approximately 400 bp downstream from the origin, the Pol III

holoenzyme is assembled and takes over replication at a highly processive speed and nature.

Pol II

DNA polymerase II, a family B polymerase, is a polB gene product also known as DinA. Pol II has 3'-5' exonuclease activity and participates in DNA repair, replication restart to bypass lesions, and its cell presence can jump from ~30-50 copies per cell to ~200-300 during SOS induction. Pol II is also thought to be a backup to Pol III as it can interact with holoenzyme proteins and assume a high level of processivity. The main role of Pol II is thought to be the ability to direct polymerase activity at the replication fork and helped stalled Pol III bypass terminal mismatches.

Pol III

DNA polymerase III holoenzyme is the primary enzyme involved in DNA replication in *E. coli* and belongs to family C polymerases. It consists of three assemblies: the pol III core, the beta sliding clamp processivity factor, and the clamp-loading complex. The core consists of three subunits: α, the polymerase activity hub, ε, exonucleolytic proofreader, and θ, which may act as a stabilizer for ε. The holoenzyme contains two cores, one for each strand, the lagging and leading. The beta sliding clamp processivity factor is also present in duplicate, one for each core, to create a clamp that encloses DNA allowing for high processivity. The third assembly is a seven-subunit ($\tau_2\gamma\delta\delta'\chi\psi$) clamp loader complex. Recent research has classified Family C polymerases as a subcategory of Family X with no eukaryotic equivalents.

Pol IV

In *E. coli*, DNA polymerase IV (Pol 4) is an error-prone DNA polymerase involved in non- targeted mutagenesis. Pol IV is a Family Y polymerase expressed by the dinB gene that is switched on via SOS induction caused by stalled polymerases at the replication fork. During SOS induction, Pol IV production is increased tenfold and one of the functions during this time is to interfere with Pol III holoenzyme processivity. This creates a checkpoint, stops replication, and allows time to repair DNA lesions via the appropriate repair pathway. Another function of Pol IV is to perform translesion synthesis at the stalled replication fork like, for example, bypassing N2-deoxyguanine adducts at a faster rate than transversing undamaged DNA.

Reverse transcriptase

Reverse transcriptase is a common name for an enzyme that functions as a RNA-dependent DNA polymerase. They are encoded by retroviruses, where they copy the viral RNA genome into DNA prior to its integration into host cells.

Reverse transcriptase has two activities:

- DNA polymerase activity: In the retroviral life cycle, reverse transcriptase copies only RNA, but, as used in the laboratory, it will transcribe both single-stranded RNA and single-stranded DNA templates with essentially equivalent efficiency. In both cases, an RNA or DNA primer is required to initiate synthesis.
- RNase H activity: RNase H is a ribonuclease that degrades the RNA from RNA-DNA hybrids, such as are formed during reverse transcription of an RNA template. This enzyme functions as both an endonuclease and exonuclease in hydrolyzing its target.

All retroviruses have a reverse transcriptase, but the enzymes that are available commercially are derived from one of two retroviruses, either by purification from the virus or expression in E. coli:

- Moloney murine leukemia virus: a single polypeptide
- Avian myeloblastosis virus: composed of two peptide chains

Both enzymes have the same fundamental activities, but differ in a number of characteristics, including temperature and pH optima. Most importantly, the murine leukemia virus enzyme has very weak RNase H activity compared to the avian myeloblastosis enzyme, which makes it the clear choice when trying to synthesize complementary DNAs for long messenger RNAs.

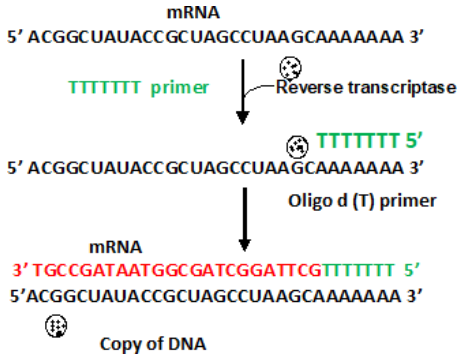


Fig. 1.7 Synthesis of cDNA on mRNA template by the action of reverse transcriptase

Reverse transcriptase is used, as you might expect, to copy RNA into DNA. This task is integral to cloning complementary DNAs (cDNAs), which are DNA copies of mature messenger RNAs. Cloning cDNAs is discussed elsewhere in more depth, but the technique is usually initiated by mixing short (12-18 base) polymers of thymidine (oligo dT) with messenger RNA such that they anneal to the RNA's polyadenylate tail. Reverse transcriptase is then added and uses the oligo dT as a primer to synthesize so-called first-strand cDNA.

Another common use for reverse transcriptase is to generate DNA copies of RNAs prior to amplifying that DNA by polymerase chain reaction (PCR). Reverse transcription PCR, usually called simply RTPCR, is a stupifyingly useful tool for such things as cloning cDNAs, diagnosing microbial diseases rapidly and a myriad of other applications. In most cases, standard preparations of reverse transcriptase are used for RTPCR, but mutated forms with relatively high thermal stability have been developed to facilitate the process.

B) Vectors:

Gene cloning vector deals with the transfer of the desirable gene into a host cell. The cell which received desired gene is call host cell.

The desired gene cannot de directly introduced in to a cell. Because the desired gene does not carry the origin of replication. Hence the desired gene has to be introduced into an autonomously replicating extrachromosomal bodies. These extrachromosomal bodies are present in bacteria, bacteriophage and viruses. These extrachromosomal bodies transport the desired DNA fragment into host cell and they are termed s cloning vector. The may plasmids or Phase or virus.

Characteristics of a cloning vectors

1. it must be small in size
2. It must be self-replicating inside host cell
3. It must possess restriction site for Restriction Endonuclease enzymes
4. Introduction of donor DNA fragment must not interfere with replication property of the vector
5. It must possess some marker gene such that it can be used for later identification of recombinant cell
6. it must possess multiple cloning site

Types of cloning vectors used in gene cloning:

1. Plasmid -psBR322
2. Bacteriophage-Lambda phage,
3. Bacterial artificial chromosome (BAC):
4. Yeast artificial chromosome (YAC):
5. Virus-SV40,
6. Cosmid vectors

Plasmid vectors: Plasmid -psBR322

Plasmids are autonomously replicating circular DNA molecules found in bacteria. They have their own origin of replication, and they replicate independently of the origins on the "host" chromosome. Replication is usually dependent on host functions, such as DNA polymerases, but regulation of plasmid replication is distinct from that of the host chromosome.

'p' indicates as a plasmid

'BR' identifies Bo-liver and Rodriguez, the two researchers who developed it

322' distinguishes those plasmids from others (like pBR 325, pBR 327, etc.) developed in the same laboratory.

The size of a plasmid is 4361 bp, and the cloning limit is 0.1-10 kb.

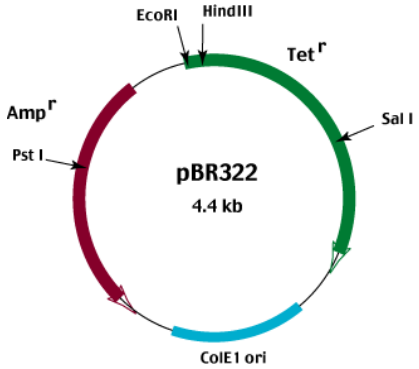


Figure 1.8. Features of plasmid pBR322. The gene conferring resistance to ampicillin (*Amp*^{r}) can be interrupted by insertion of a DNA fragment into the PstI site, and the gene conferring resistance to *tetr*acycline (*Tet*^{r}) can be interrupted by insertion of a DNA fragment into the BamHI site. Replication is controlled by the ColE1 origin.

A plasmid that was widely used in many recombinant DNA projects is pBR322.

It replicates from an origin derived from a colicin-resistance plasmid (ColE1). This origin allows a fairly high copy number, about 100 copies of the plasmid per cell.

Plasmid pBR322 carries two antibiotic resistance genes, namely ampicillin resistance gene *Amp*^{r} and *tetr*acycline resistance gene *Tet*^{r} each derived from different transposons.

There are about 20 restriction sites in this plasmid. The *Tet*^{r} gene has 6 restriction site and *Amp*^{r} gene has 3 restriction site.

Use of the *Amp*^{r} and *Tet*^{r} genes allows for easy screening for recombinants carrying inserts of foreign DNA. For instance, insertion of a restriction fragment in the *Bam*HI site of the *Tet*^{r} gene inactivates that gene. One can still select for *Amp*^{r} colonies, and then screen to see which ones have lost *Tet*^{r}.

Advantages of pBR322:

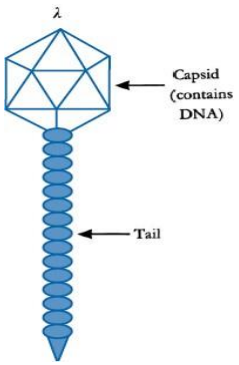
1. Small size (~ 4.4 kb) enables easy purification and manipulation.
2. Two selectable markers (amp and tet) allow easy selection of recombinant DNA.
3. It can be amplified up to 1000-3000 copies per cell when protein synthesis is blocked by the application of chloramphenicol.

Bacteriophage-Lambda phage

The most extensively studied bacterial virus used for DNA cloning is bacteriophage λ (Lambda).

The λ phage virion has a head region containing the viral DNA genome, and a tail which functions in infecting its host, the E. coli

The wild-type A virus particle or virion contains a 50 kb liner double-stranded DNA as its genome which is packaged within a protein coat. When a virion attaches itself to the host bacterial cell, the coat protein is discarded and A DNA is injected into the cell.



At the extreme termini of the λ DNA are overhanging 5′ ends which are 12 nucleotides long, and complementary in base sequence. These large 5′ overhangs can base- pair, and are effectively sticky ends, similar to, but more cohesive than the small sticky ends generated by some restriction nucleases (*Eco* RI).

Due to this cohesive property such sequence is called the cos sequence. Once inside the bacterial cell, the cos sequences base-pair and sealing of the nicks by cellular ligases results in the formation of a double-stranded circular DNA. The wild type DNA contained several cleavage sites for the commonly used Restriction enzymes, so it is not suitable for a vector. Because the Restriction enzyme cuts the DNA molecules into several small fragments. For this reason, the wild type phase DNA is not used directly as a cloning vector. Derivatives of the wild type have been produced and used as cloning vector.

There are two types of lambda cloning vectors.

(a) Lambda Insertion Vectors:

In this case a large segment of the non-essential region has been deleted, and the two arms ligated together. An insertion vector possesses at least one unique restriction site into which new DNA can be inserted. The size of the DNA fragment that an individual vector can carry depends on the extent to which the non-essential region has been deleted, e.g.; lambda-gtl0, lambda- ZAP11.

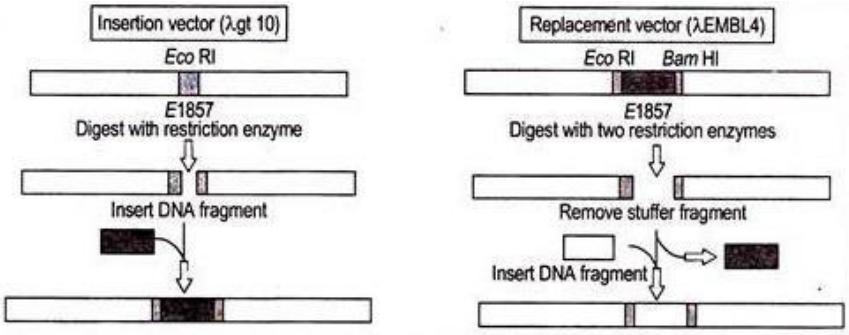


Fig. Scheme used for cloning in λ insertion and λ replacement

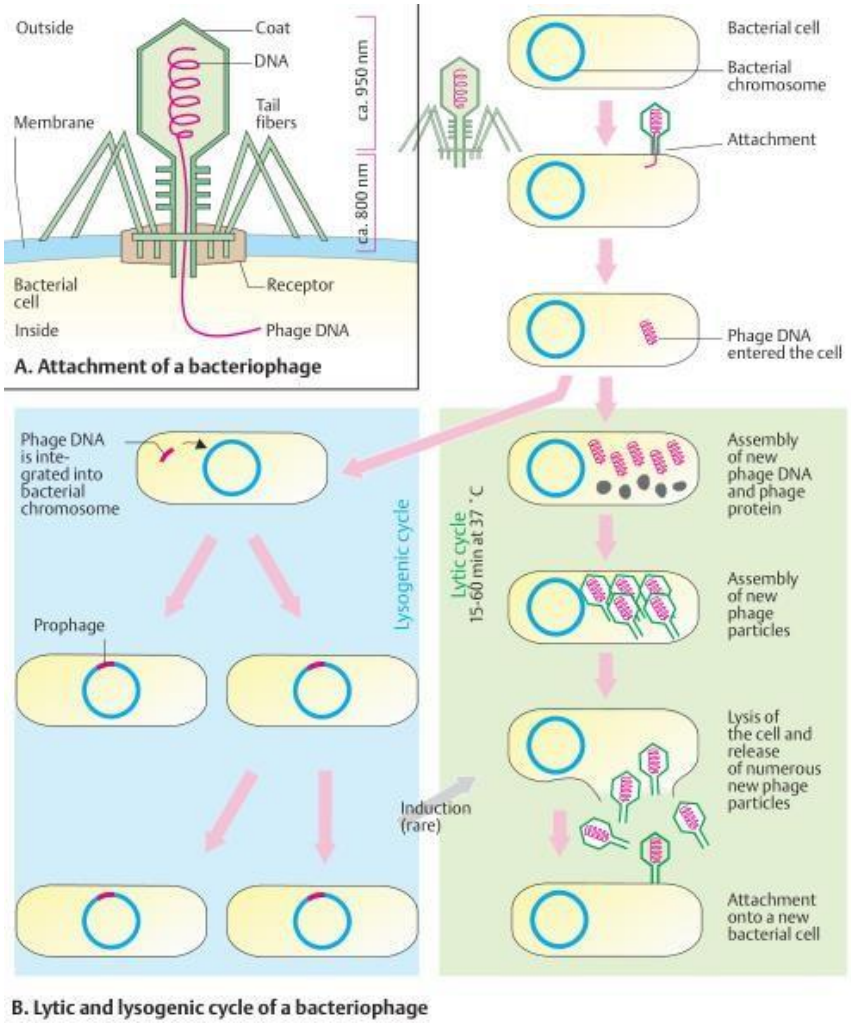
(b) Lambda Replacement Vectors:

These vectors have two recognition sites for the restriction endonucleases. These sites flank a segment of DNA that is replaced by the DNA to be cloned. Often the replaceable fragment (or stuffer fragment) carries additional restriction sites that can be used to cut it up into small pieces so that its own reinsertion during a cloning experiment is very unlikely.

Replacement vectors are generally designed to carry large pieces of DNA than insertion vectors can handle e.g., lambda- EMBL, lambda-GEMll, etc.

(i) The Lytic Cycle:

Once inside the bacterial cell, the cos sequences base-pair and sealing of the nicks by cellular ligases results in the formation of a double-stranded circular DNA. After that the λ DNA can undergo one of two alternative pathways.



(ii) The Lysogenic Cycle:

The lambda genome possesses a gene att which has a homolog in the E. coli chromosome. Apposition of the two att genes can cause recombination between the λ and E. coli genomes

and subsequent integration of the λ DNA within the E. coli chromosome. In this condition the phage DNA is called provirus and the host cell as lysogen.

The phage DNA can remain stably integrated for long periods, but it has the capacity for excision from the host chromosome and entry into the lytic cycle. The genes controlling the lysogenic cycle of the phage are located in a central segment of the λ genome.
However, the decision of the phage to enter either lytic or lysogenic cycle is controlled by two regulatory genes; cl and cro which are mutually antagonistic. In the lytic state, the cro gene dominates, causing the repression of cl.
Whereas, in the lysogenic state, the cl dominates, and suppresses transcription of some λ genes including cro. Normally, the lysogenic state is favoured and the phage genome is replicated along the host chromosomal DNA.
In the DNA genome, λ genes encoding the head, tail proteins and proteins responsible for lytic and lysogenic growths are clustered in discrete regions of the ≈ 50kb viral genome. Genes that are not involved in lytic pathway and that are involved in lysogenic pathway are not essential for the use of the λ phage as a cloning vector. So, these genes may be removed from the viral DNA and are replaced with other DNA fragment of interest. In this way foreign DNA up to ≈ 25 kb can be inserted into the λ genome.
The recombinant λ phages are able to transform E. coli cells at high efficiency. It is achieved by developing an in vitro packaging system which mimicked the way in which wild-type λ DNA is packaged in a protein coat, resulting in high infection efficiency. In this way several important cloning vectors have been developed by modifying the lambda phage genome.

Virus SV40 Vectors:

SV40 is a spherical virus with a circular, double-stranded 5,243 bp chromosome, which encodes 5 proteins, viz., small-T, large-T (both early proteins), VP1, VP2 and VP3 (VP = virion protein), has an origin of replication (about 80 bp) and is complexed with histones to form chromatin. Large-T is essential for viral replication, while VP1, VP2 and VP3 form the viral capsid.
In laboratory, it is multiplied in cultured kidney cells of African green monkey; infected cells lyse after 4 days releasing upto 10^5 virions ⁄cell. SV40 genome has been used to develop mainly the following three types of vectors:
(1) transducing vectors, (2) plasmid vectors and (3) transforming vectors.

SV40 Transducing Vectors:

These vectors produce viral particles after infecting monkey cells. They must have the following three features:

1. the SV40 origin including the surrounding region containing the transcriptional regulatory signals {i.e., regions at which splicing and polyadenylation occur),

2. total size (including, that of the DNA insert) between 3,900 bp and 5,300 bp for packaging into virions, and
3. genes encoding large-T, VP1, VP2, and VP3. This leaves very little room for DNA inserts. But the genes encoding the necessary proteins, viz., large-T, VP1, VP2 and VP3, can be present in another virus or within the host genome. This flexibility is used to advantage for solving the size and selection problems.

Late Replacement Vectors:

The region encoding VP1, VP2 and VP3 may be replaced in the vector by DNA insert; such a vector is called late-region replacement vector, e.g., SVGT-5 (an improved SV40 vector). A vector of this type contains the following: (1) the origin of replication, (2) the regions at which splicing and polyadenylation occur, and (3) the entire early region of SV40 genome.

Such a vector is used for infection of host cells in conjunction with another virus, called helper virus, which has the VP1, VP2 and VP3 genes intact but has a defective large-T gene (a gene in the early region) gene. The large T function is intact in and provided by the late replacement vector.

Therefore, in this case, only those host cells that are infected by both the vector and the helper virus will lyse and produce virions since cells infected by either the vector or the helper virus alone will not support packaging or replication (respectively) of the virus. This feature is very useful since all the plaques formed on monkey cell monolayers contain the vector.

Late-region replacement vectors have been used to study post-transcriptional RNA processing and stability. The presence of an intron in the late-region primary transcript was necessary in some (but not in other) cases, for the production of cytoplasmic mRNA, the reasons for this being unknown.

Early Replacement Vectors:

Alternatively, the essential genes missing from the vector may be present within the genome of host cells. For example, COS (CV-1, Origin of SV40; CV-1 is a monkey cell line) cell line of African green monkey kidney cell cultures contains in its genome the gene for large-T of SV40.

Therefore, a vector having the origin of replication and genes for VP1, VP2 and VP3 will replicate and produce virions in COS cell line cells. In such a case, no helper virus is required. Since in such a vector the early genes (large-T gene) are replaced by the DNA insert, it is called early-region replacement vector.

The chief advantages of both late- and early-region replacement SV40 vectors are as follows.

1. The recombinant DNA produces virions, which introduce the DNA into host cells by infecting them like SV40 virions.
2. The recombinant DNA replicates to a high copy number, which is a distinct advantage.

These vectors have the following two limitations:

1. the expression of transgenes is transient in the infected cells, and
2. the maximum size of DNA insert is -2.5 kb.

SV40 Plasmid Vectors:

These vectors replicate in monkey cells but do not get packaged into virions. They contain the origin of replication and the large-T encoding gene (large-T gene is not necessary for multiplication in COS cells). Obviously, there is no size limit on such vectors, and some of them are E. coli and monkey shuttle vectors, e.g., pSV2, pSV3, etc.. These vectors produce high copy number per COS cell; this makes the host cells inviable.

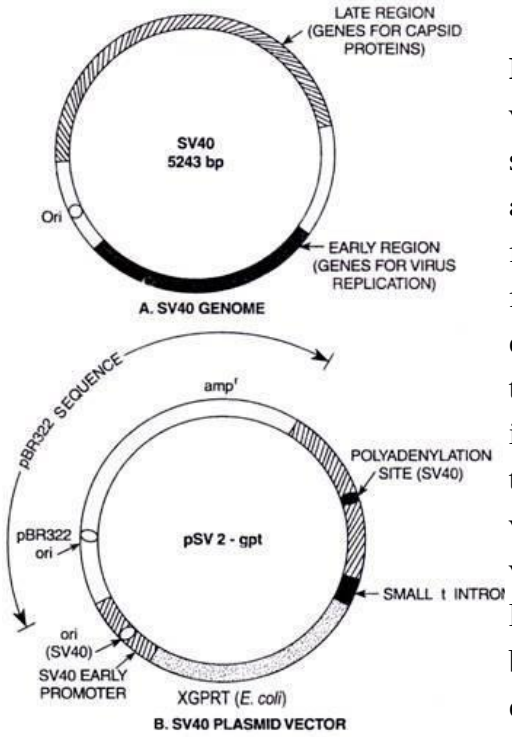


Fig. 1.9: SV40-based plasmid vector. A. SV40 genome. B. A shuttle vector having origin and amp' gene from pBR322 (for function in E. coli), and the following SV40 segments: the origin and the neighbouring transcriptional control sequences including early promoter, and the polyadenylation site. This vector functions as a plasmid vector in host cells having the large-T gene, e.g., COS cell line, but as a nonreplicative vector in other host cells.

Therefore, permanent COS cell lines having recombinant DNA are not obtained, but transient expression of cloned genes can be analysed. The shuttle vectors are used to propagate the recombinant DNA in E. coli, which are then introduced into monkey cells to study the expression of DNA inserts. Plasmid vectors can be constructed by using the origin and the early region of polyoma virus in the place of those of SV40.

pSV (plasmid simian virus) vectors are constructed by incorporating the SV40 sequences into pBR322. The bacterial XGPRT gene (or mouse DHFR gene or bacterial neomycin phosphotransferase gene) integrated after the SV40 early promoter sequence serves as selectable marker when the vector is transferred into mammalian cells.

pRSV series of vectors are derivatives of pSV vectors. In these vectors, the SV40 early promoter sequence is replaced by a 524 bp long fragment from the long terminal repeat (LTR) sequence from the retrovirus Rous sarcoma virus (RSV); this sequence contains the retroviral promoter sequence. The retroviral promoter is more powerful than the SV40 promoter in various cell types.

Plasmid vectors are unstable in monkey cells, and are generally used for transient transfection only. These vectors can be stably maintained if the large-T function is provided by COS cells and the vector has a selectable marker, say, E. coli neo gene, and the host cells are maintained under selection environment, in this case on G-418. Plasmid vectors also integrate in the host cell genome with a frequency of 10^{-5} to 10^{-3}; this yields stable transfection.

Plasmid vectors have been mainly used to investigate sequences involved in transcriptional and post-transcriptional regulation. For example, these studies permitted the discovery of SV40 enhancer sequences and the analysis of its properties. Stable transfectant COS cell lines have been produced by using one of the following two strategies. In one strategy, the gene encoding large T protein, present in the COS cell line, is a temperature-sensitive mutant.

But in the second strategy, the wild type allele of large T gene is placed under the control of metallothionein gene promoter. In both these cases, the SV40-based plasmid is stably maintained as an episome at a low copy number. But they do permit the activity of large T protein to be increased. This leads to an increase in the number cf copies of the plasmid and in the level of expression of the trangene contained in the plasmid.

Cosmids Vectors

Cosmids are essentially plasmids that contain a minimum of 250 bp of X DNA, which includes the following sequences from phage X genome:
(1) the cos site (the sequence yielding cohesive ends) and (2) sequences needed for binding of and cleavage by terminase so that under appropriate conditions they are packaged in vitro into empty A, phage particles. A typical cosmid has (1) replication origin, (2) unique restriction sites and (3) a selectable markers from a plasmid (Fig. 2.18). Cosmid vectors are constructed using recombinant DNA techniques.

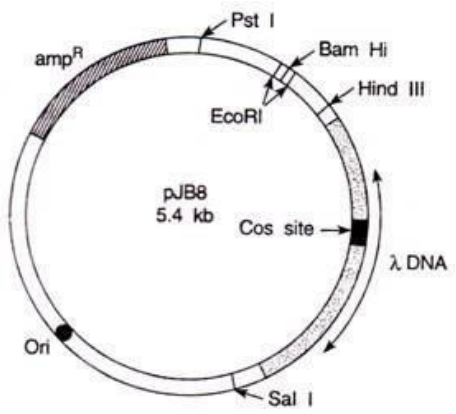


Fig. 1.10 A typical cosmid vector containing plasmid modules and a λ segment having the cos site, and terminase binding and cleavage sequences

The cosmid vectors are opened by the appropriate restriction enzyme at a unique site, are then mixed with DNA inserts prepared by using the same enzyme and annealed. Among the several types of products, long cancatemers are present, which are the appropriate precursors for packaging in λ particles.

This procedure selects for long DNA inserts since for packaging the distance between two cos sites must be between 38 and 52 kb. The DNA fragments used for cloning are usually produced by partial digestion with a restriction enzyme.

This is because complete digestion almost always produces fragments that are too small for cloning in a cosmid. Cosmids can accommodate upto 40 kb long DNA inserts (Table 2.10). Packaged cosmids infect host cells like X particles, but once inside the host they replicate and propagate like plasmids. Because of the size limitation, only recombinant cosmids will be packaged in λ phage heads. The transformed bacterial cells are selected on the medium containing the concerned selection agents.

The typical features of cosmids are as follows:

(1) They can be used to clone DNA inserts of upto 40 kb. (2) They can be packaged into λ particles, which infect host cells; this is many-fold more efficient than plasmid transformation. (3) Selection for recombinant DNA is based on the procedure applicable to the plasmid making up the cosmid. (4) Finally, these vectors are amplified and maintained in the same manner as the contributing plasmid.

Cosmids are particularly attractive for construction of genomic libraries of eukaryotes since they can be used for cloning large fragments. Since partial digests are used, two or more genomic fragments may join together in the ligation reaction.

Techniques used in Genetic Engineering

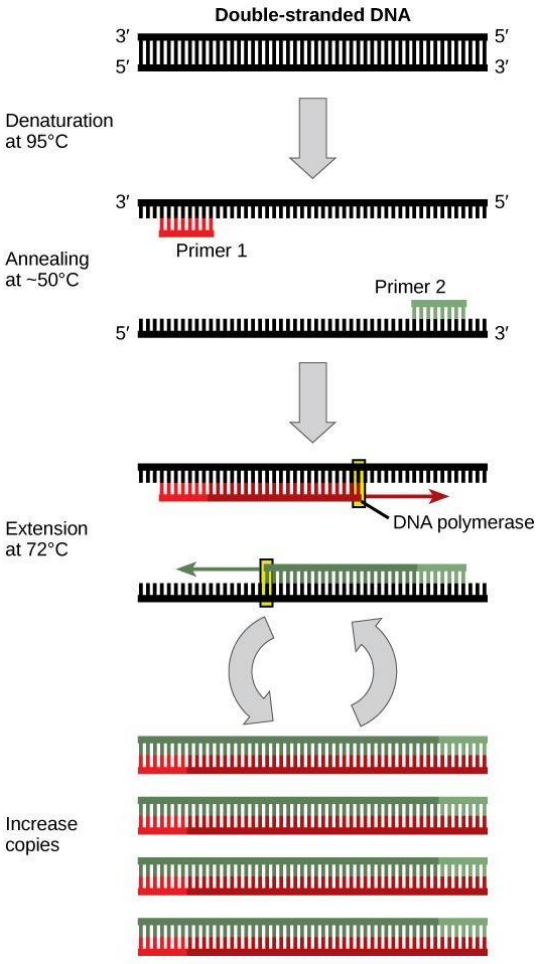
Polymerase Chain Reaction (or PCR)

The **polymerase chain reaction** (PCR) is the most powerful technique that has been developed recently in the area of recombinant DNA research and is having an impact on many areas of molecular cloning and genetics. With this technique a target sequence of DNA can be amplified a billion fold in several hours. This procedure has been applied to forensic analysis where minute samples of DNA was isolated from blood at a crime scene to determine if an individual was actually at the location of the crime. Most recently DNA from mummies and fossil tissue has been analyzed to determine ancient evolutionary relationships.

PCR is a DNA polymerase reaction and as with any polymerase reaction it requires a DNA template and a free 3'-OH. The **template is provided by the DNA sample to be amplified and the free 3'-OH are provided by site-specific oligonucleotide primers**. The primers are complementary to each of the ends of the sequence that is to be amplified. The three steps of the reaction are:

1. Denaturation - the DNA is heated usually to 95^{o}C to render it single-stranded

2. Annealing - the two primers bind the appropriate complementary strand; the temperature for this step varies depending on the of size of the primer and its homology to the target DNA



3. Primer Extension - DNA polymerase extends the primer by its polymerase activity; this is done at a temperature optimal for the particular polymerase that is used; currently the most popular enzyme for this step is *Taq* polymerase, the DNA polymerase from the thermophilic ("heat-loving) bacteria *Thermus aquaticus*; the extension is performed at 72^{o}C

These steps are repeated from 28-35 times. Since the reaction is essentially exponential and since each cycle is about 5 minutes, a large quantity of DNA can be produced for analysis in as little as several hours.

The first description of the PCR reaction used the DNA polymerase I enzyme from *E. coli*, and the reaction was moved manually between the different temperatures. Because the *E. coli* enzyme is heat sensitive, its activity was killed during the denaturation step at 95oC. Therefore a new aliquot of the enzyme had to be added with each cycle.

The purification, and ultimately the cloning, of the DNA polymerase from *T. aquaticus* made the reaction much simpler. This organism lives in hot springs that can be near boiling and since it can complete its life cycle at these temperatures, its DNA polymerase would need to be able to maintain its functions after being exposed to these temperatures. This property makes it highly favorable for the PCR reactions because the denaturation step is performed at 95^{o}C. Thus a new enzyme aliquot is not required for each cycle. More recently, DNA polymerases have been isolated from organisms that reside in the vicinity of the thermal vents that are present in the oceans at the junction of two tectonic plates. These enzymes are even more heat stable and may eventually replace the T. *aquaticus* enzyme for the PCR reaction.

Finally, this procedure has been automated by the development of thermal cyclers. These instruments have the capability of rapidly switching between the different temperatures that are required for the PCR reaction. Thus the reactions can be set up, placed in the thermal cycler and the technician can return several hours later and obtain the product and proceed from that point.

GEL ELECTROPHORESIS

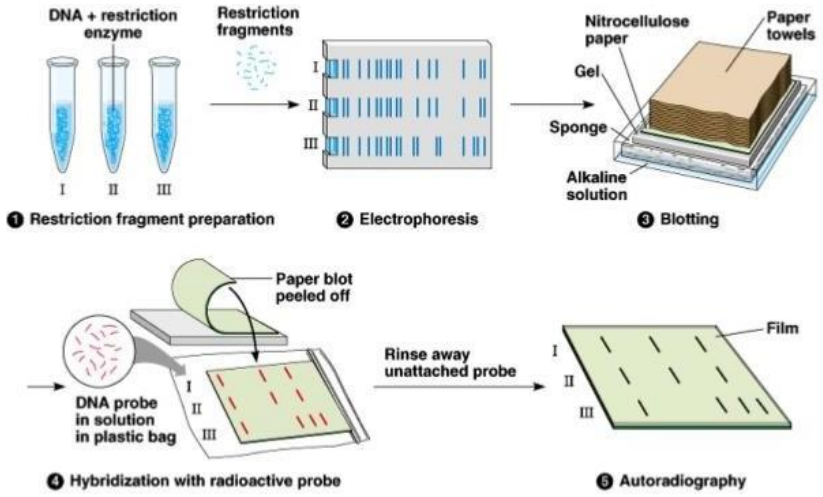
Gel electrophoresis is a laboratory method used to separate mixtures of DNA, RNA, or proteins according to molecular size. In gel electrophoresis, the molecules to be separated are pushed by an electrical field through a gel that contains small pores. The molecules travel through the pores in the gel at a speed that is inversely related to their lengths. This means that a small DNA molecule will travel a greater distance through the gel than will a larger DNA molecule.

As previously mentioned, gel electrophoresis involves an electrical field; in particular, this field is applied such that one end of the gel has a positive charge and the other end has a negative charge. Because DNA and RNA are negatively charged molecules, they will be pulled toward the positively charged end of the gel. Proteins, however, are not negatively charged; thus, when researchers want to separate proteins using gel electrophoresis, they must first mix the proteins with a detergent called sodium dodecyl sulfate. This treatment makes the proteins unfold into a linear shape and coats them with a negative charge, which allows them to migrate toward the positive end of the gel and be separated. Finally, after the DNA, RNA, or protein molecules have been separated using gel electrophoresis, bands representing molecules of different sizes can be detected.

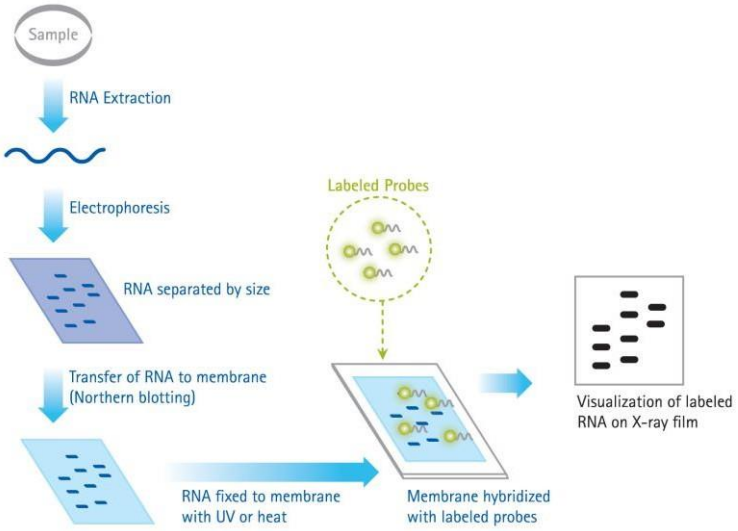
Southern and Northern Hybridiazation Analysis

The hybridization or binding of a clone to DNA or RNA provides important information regarding the structure and the expression of the gene.

SOUTHERN HYBRIDIZATIONS



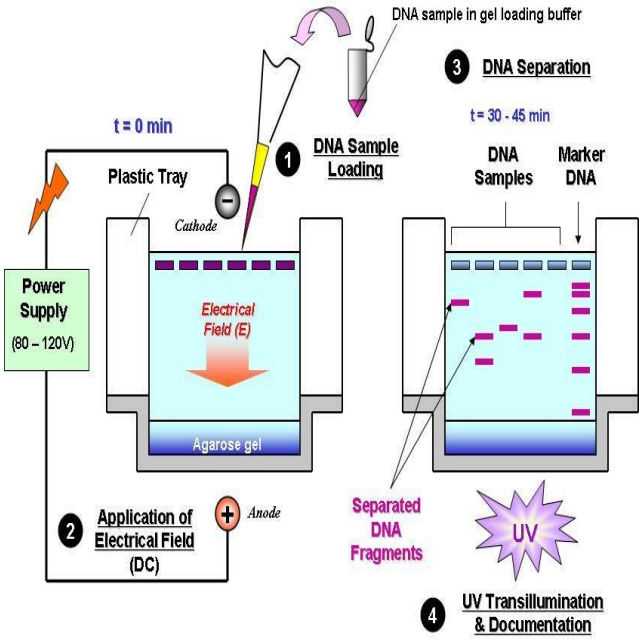
Southern hybridizations involve the binding of a radioactive probe to a DNA molecule that is immobilized on a membrane filter. After a series of washes the filter is used to expose a piece of X-ray film. After exposure, the film is developed, and a band appears where the hybridization occurs. Each of these hybridizations will represent the size of fragments which contain sequences that are complementary to the probe. By hybridizing the probe to a series of restriction digestions of the DNA of interest, the structure of the gene can be analyzed.



Let's assume that your gene does not contain any internal*EcoRI* sites. Then if you hybridize a clone (for example a cDNA clone for some gene) to an *EcoRI* digestion of your DNA and you see two fragments, then you can conclude that the species you are analyzing has two copies of the gene. Normally, you do not know *a priori* what restriction enzyme sites are located in the gene, so a series of digestions and hybridizations are performed. If five out the six experiments reveal two bands, and the probe hybridizes to three fragments of DNA digested with a sixth, then you can conclude that two genes exist and one of these genes contains a restriction cleavage site for the third enzyme.

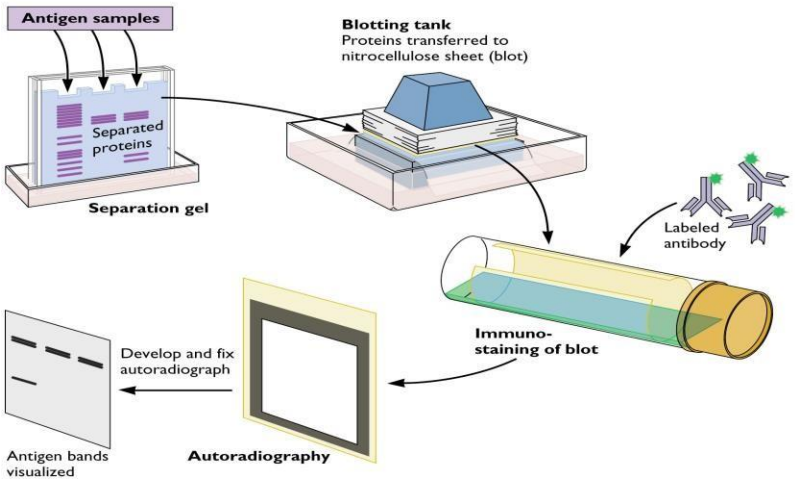
Northern hybridizations

Northern hybridizations involve a radioactive probe and RNA that is immobilized on a filter membrane. The hybridization is between complementary bases in the RNA and the probe. These hybridizations are performed to study the expression of the gene. RNA is typically isolated from different tissues and from different developmental stages of species. After electrophoresis, the RNA is transferred to the membrane and probed. If, for example, the probe hybridizes only to RNA from heart tissue after the individual reaches adult age, it can be concluded that the gene is only expressed in the adult heart.



In general, genes can be expressed constitutively, temporally or spatially. **Constitutive expression** implies that the gene is expressed at all times. mRNA for genes that exhibit **spatial expression** are only found in specific tissues of the organism. If a gene is only expressed during a specific time in development it is said to exhibit **temporal expression**. Combinations of expression patterns are also possible. For example, a gene that is always expressed in a leaf tissue is exhibiting constitutive, spatial expression

Western Blotting



Western blot analysis can detect **one** protein in a mixture of any number of proteins while giving you information about the size of the protein. It does not matter whether the protein has been synthesized in vivo or in vitro. This method is, however, dependent on the use of a high-quality antibody directed against a desired protein. So you must be able to produce at least a small portion of the protein from a cloned DNA fragment. You will use this antibody as a probe to detect the protein of interest.

Western blotting tells you how much protein has accumulated in cells. If you are interested in the rate of synthesis of a protein, **Radio-Immune Precipitation** (RIP) may be the best assay for you. Also, if a protein is degraded quickly, Western blotting won't detect it well; you'll need to use (RIP).

1. **Separate the proteins** using SDS-polyacrylamide gel electrophoresis (also known as **SDS-PAGE**). This separates the proteins by size.
2. **Place a nitrocellulose membrane on the gel** and, using electrophoresis, drive the protein (polypeptide) bands onto the nitrocellulose membrane. You want the negative charge to be on the side of the gel and the positive charge to be on the side of the nitrocellulose membrane to drive the negatively charged proteins over to the positively charged nitrocellulose membrane. This gives you a nitrocellulose membrane that is imprinted with the same protein bands as the gel.

One thing to be aware of is that proteins bind better to nitrocellulose at a low pH. You may need to go through some trial-and-error to find the optimal pH. You also need to be sure there are no air bubbles between the nitrocellulose and the gel or your proteins will not transfer.

3. Incubate the nitrocellulose membrane with a primary antibody. The primary antibody, which is the specific antibody mentioned above, sticks to your protein and forms an antibody-protein complex with the protein of interest.

4. Incubate the nitrocellulose membrane with a secondary antibody. This antibody should be an antibody-enzyme conjugate. The secondary antibody should be an antibody against the primary antibody. This means the secondary antibody will "stick" to the primary antibody, just like the primary antibody "stuck" to the protein. The conjugated enzyme is there to allow you to visualize all of this. It's kind of like a molecular flare stuck on the antibodies so you can visualize what s going on.

5. To actually see your enzyme in action, you'll need to incubate it in a reaction mix that is specific for your enzyme. If everything worked properly, you will see bands wherever there is a protein-primary antibody-secondary antibody-enzyme complex, or, in other words, wherever your protein is.

6. Put x-ray film on your gel to detect a flash of light, which is given off by the enzyme. The reaction usually runs out in about an hour.

UNIT- II

1. Construction of rDNA
2. c-DNA libraries and Genomic libraries
3. Transgenesis and Transgenic animals (Transgenic cattle, sheep, pig and fish)
4. Cloning and cloned animals (Dolly sheep)
5. DNA fingerprinting.

1. Construction of rDNA

- Single chimeric DNA formed by combining two or more different fragments of DNA from diverse organisms is generally called as recombinant DNA and the method applied to create recombinant DNA is called recombinant DNA technology.
- The organism, from which the candidate DNA is isolated, is called **Donor organism**. The organism which will accept the foreign gene is called **Host organism**.
- Genetic material from one organism is selected and then artificially introduced to a host organism. if the foreign recombinant DNA integrates into the host genome, it gets replicated along with the genome and then express the foreign protein.
- Paul Berg, Herbert W. Boyer and Stanley N. Cohen are the pioneers of recombinant DNA technology (early 1970).
- A hybrid of the *SV40* mammalian DNA virus genome and phage λ was one of the recombinant DNA molecules to be first engineered.

There are three approaches to make recombinant DNA:

1. Transformation
2. Non- bacterial transformation/transfection
3. Phage introduction/transduction

1. Transformation:
Transformation is direct uptake of exogenous DNA via cell membrane leading to incorporation into the host DNA. It is commonly occurred in bacteria. Transformation requires different tools of molecular biology to insert foreign DNA into the host. For example, **vector** to carry the foreign DNA to the host; **restriction enzymes** to cut the DNA in specific site; **ligase** to join two DNA molecule etc.

2. Non-bacterial transformation/transfection:
The process of foreign DNA uptake by host cell driven by mechanical or chemical factors is classified under non-bacterial transformation, also termed as transfection. Different methods of non-bacterial transformation are microinjection, liposome mediated transformation, biolistics etc.

3. Phage introduction/transduction:
Phage vector is used to carry and replicate foreign DNA inside the bacterial host system. The phage DNA inserts into the host chromosome by recombination. Phage *λ* had short regions of single-stranded DNA with complementary base sequences called "cohesive" (*cos*) sites. Base pairing between the complementary *cos* sites allows the linear genome to form a circle within the host bacterium. Circularized viral genome can be integrated into the bacterial genome by homologous recombination between *attP* site of viral genome and *attB* site of bacterial genome.

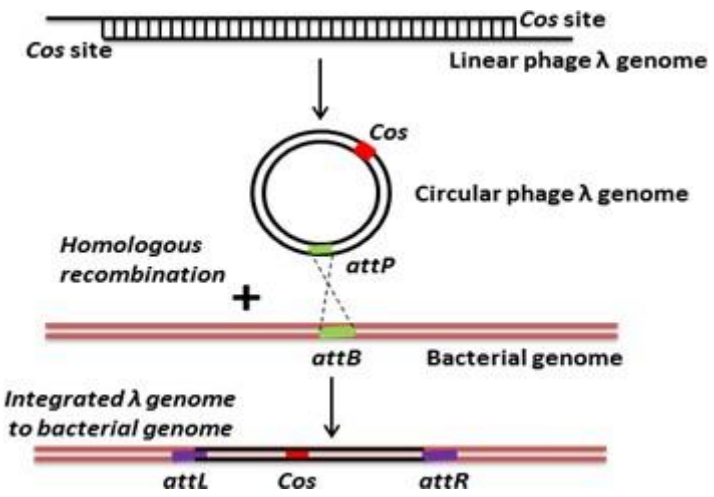


Fig 2.1: Bacteriophage λ genome circularization and recombination with bacterial genome

Methods involved in Recombinant DNA Technology :

Molecular cloning is a process for creating recombinant DNA and generally involves the following steps:

(1) Selection of a cloning vector
(2) Selection of a host organism
(3) Preparation of a vector DNA
(4) Preparation of DNA to be cloned
(5) Creation of recombinant DNA vector (having foreign DNA)
(6) Introduction of recombinant vector into host organism
(7) Selection of clones having insert vector.
(8) Screening and multiplication of recombinant clones with desired DNA inserts.

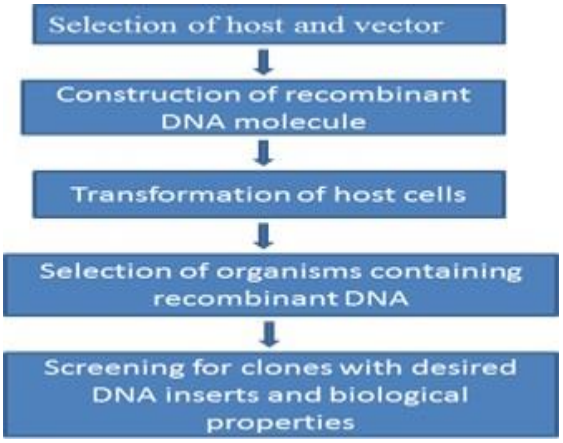


Fig 1-2.2: Steps in Gene Cloning

1-2.2.1 Choice of host organism:

A good host should have the following properties:

- Easy to grow and transform.
- Do not hinder replication of recombinant vector.
- Do not have restriction and methylase activities.
- Deficient in recombination function so that the introduced recombinant vector is not altered.
- Easily retrievable from the transformed host.

Various hosts are used in rDNA technology depending on the goal: For example bacteria, yeast, plant cells, animal cells, whole plants and animals.

Prokaryotic systems such as *E. coli* are commonly used due to various advantages like,

- They have-Well studied expression system,
- Compact genome,
- Versatile,
- Easy to transform,
- Widely available, and
- Rapid growth of recombinant organisms with minimal equipment.

Only disadvantage is that they lack post-translational modification (PTMs) machinery required for eukaryotic proteins.

Eukaryotic systems are difficult to handle in contrast to bacterial hosts. They are favoured for expression of recombinant proteins which require post translational modification and only if they can grow easily in continuous culture.

Choice of vector:

Vector is an autonomously replicating (inside a host cell) DNA molecule designed from a plasmid or phage DNA to carry a foreign DNA inside the host cell. Transformation vectors are of two types:

- Cloning vector is used increasing the number of copies of a cloned DNA fragment.
- Expression vector is used for expression of foreign gene into a protein.
- If a vector is designed to perform equally in two different hosts, it is called a shuttle vector.

Properties of an ideal vector: A good vector should have the following characteristics:

- Autonomously replicating i.e. should have ori (origin of replication) region.
- Contain at least one selectable marker *e. g.* gene for antibiotic resistance.
- May contain a scorable marker (β -galactosidase, green fluorescent protein etc.)
- Presence of unique restriction enzyme site.
- Have multiple cloning sites.
- Preferably small in size and easy to handle.
- Relaxed control of replication to obtain multiple copies.
- Presence of appropriate regulatory elements for expression of foreign gene.
- High copy number

The selection of a suitable vector system depends mainly on the size limit of insert DNA and the type of host intended for cloning or expression of foreign DNA.

List of different vectors

Plasmids are circular DNA molecules that exist independently of chromosomal DNA and can replicate autonomously. Plasmids carry one or more genes which mostly code for useful characteristic of host. All plasmids have sequence that can act as origin of replication. Plasmids of different sizes and possessing different copy number are present.

Phage vectors are consist of mainly DNA molecule (sometimes RNA); that carrys large number of genes and are surrounded by a protein coat called as capsid. They can be used as vehicles for carrying DNA insert after modification to remove pathogenic genes and minimizing the size.

Cosmids are hybrid between a phage DNA and bacterial plasmid. They have *cos* sites which are essentially required for packaging lambda (λ) into phage protein coat. They can carry large DNA insert.

Fosmids are cosmid like plasmid but they are based on F-plasmid.

Phagemids are plasmids having a part of M13 genome.

Artificial chromosomes are artificially constructed DNA construct used for transferring DNA.

Preparation of vector DNA:

The vector DNA is cleaved by restriction endonucleases at the site where foreign DNA is desired to be inserted. The restriction enzyme is selected to generate a configuration at the cleavage site compatible with the ends of the foreign DNA. This can be achieved either by cleaving the foreign DNA and vector DNA with the same restriction enzyme or by adding adaptors/ linkers to both the ends of the insert DNA.

Preparation of DNA to be cloned:

DNA to be cloned can be obtained by:
1. Cutting using restriction enzyme from genomic or organellar DNA,
2. PCR based amplification,
3. Chemical synthesis.

The DNA to be cloned is isolated and treated with restriction enzymes to generate random fragments with ends capable of being linked to those of the vector. While choosing the restriction enzyme to cut the desired gene, care should be taken so that the restriction enzyme does not cut in the middle of the gene, but only at the ends. PCR based methods are used to obtain DNA segments, using either genomic DNA or mRNA as template sequences through reverse transcription. Short length sequences can be artificially

synthesized *in vitro*. If necessary, linkers or adapters containing desired restriction sites are added to create the ends which are compatible with the vector. The complementary sticky ends result in an efficient ligation due to the formation a stable structure.

Creation of recombinant DNA by ligation

- The vector DNA, foreign DNA and DNA ligase enzyme are added together at appropriate concentrations which results in the covalent linkage between the ends of DNA fragments.
- DNA ligase recognizes the ends of linear DNA molecules and gives a complex mixture of DNA molecules with randomly joined ends.
- The resulting recombinant DNA vector is then introduced into the host organism.

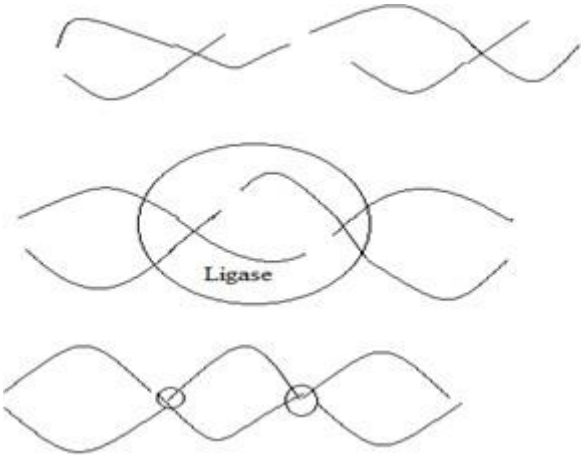


Fig 2.2.5: Ligation using ligase enzyme

- In addition to desired recombinant DNA, complex mixture containing self ligated vector DNA, foreign DNA linked with other sequences and several other combinations of vector and foreign DNA also appear in the reaction mixture.
- Sorting of the complex mixture is done by agarose gel electrophoresis based on size of the recombinant vector.

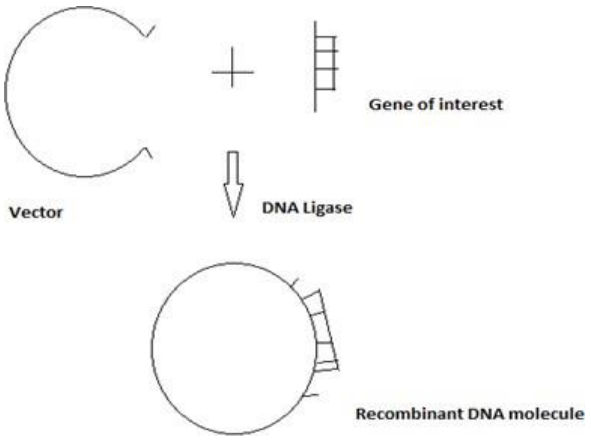


Fig 2.2.5.1: Preparation of recombinant DNA

1-2.2.6 Introduction of recombinant DNA into host organism:

- For the propagation of a cloned gene, the recombinant DNA molecules have to be introduced into a host.
- Numerous methods of gene transfer are available to meet the diverse requirement and compatibility with the host (e.g. transformation, transduction, transfection, electroporation etc.).

Transformation is the process in which microorganisms are able to take up the DNA from their surrounding via plasma membrane and express it. Cells should be competent to take up the foreign DNA.

- DNA transfer into mammalian cells cultured in vitro using non-viral vectors is termed as **transfection**.

Transduction is the process of transfer of DNA molecule using viruses.

- Both transformation and transfection requires preparation of the cells through a specific growth condition and chemical treatment process that varies with the specific species and cell types to be used. For example – Calcium chloride is used for preparation of competent *E.coli*cells.
- Electroporation uses electrical pulses to create transient holes in the cell membrane through which DNA is translocated across the cell membrane. Cell wall has to be previously removed in plants to increase the rate of transfer. Electroporation is usually done by two methods:
- High voltage for short time,
- Low voltage for long time.
- Electroporation and transduction are very efficient methods to transfer DNA into cells.

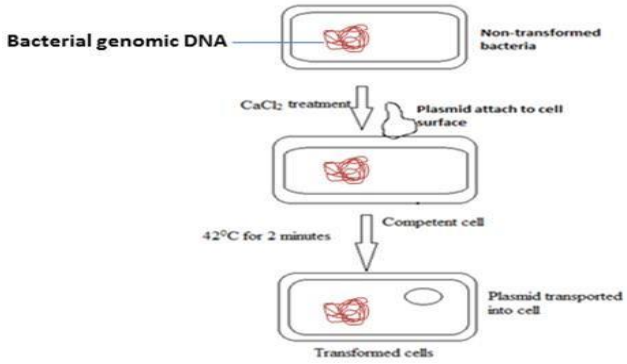


Fig 1-2.2.6: Preparation of competent cells

Selection of host cells/organism containing vector sequences:

Selection of the transformed cells from the non-transformed population is done by using selectable marker genes that confers resistance to antibiotics. Hence, cells only having the vector with the resistance gene for the antibiotic would grow in the selection media containing the antibiotic (ampicillin, tetracycline etc.); while the non-transformed cells would die.

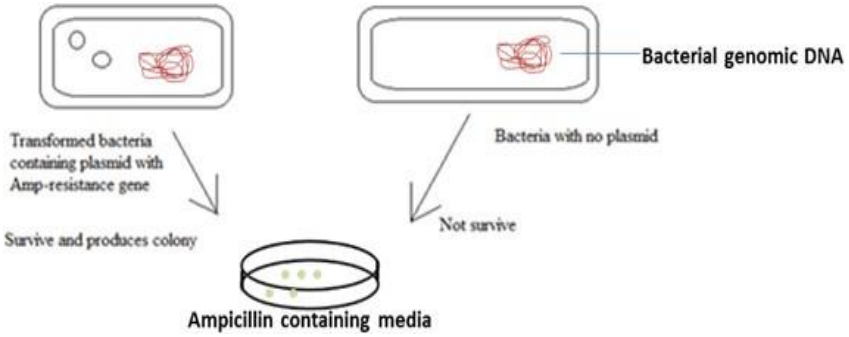


Fig 1-2.2.7: Selection of transformed cells

Insertional inactivation of antibiotic gene can also be used for the selection of recombinant cells.

- A vector is chosen where restriction sites are available for cloning within the antibiotic gene. Insertion of a foreign gene in the restriction site will lead to the loss of activity of the selectable marker (antibiotic) gene. For example-pBR322 have several restriction sites. *BamH1* cuts at a one position within genes that code for tetracycline resistance. Thus recombinant pBR322 carrying foreign DNA at *BamH1* site will not confer resistance to tetracycline, but are still resistant to ampicillin, which remains elsewhere.
- These recombinant cells are selected by replica plating method. The transformed cells are first plated on ampicillin containing medium and after the selection of transformed from non-transformed; the colonies are replica plated on medium containing tetracycline for screening of recombinant clones. After incubation, the viable colonies carrying pBR322 without DNA insert will appear and the positions in plate where the non-viable recombinant clones are present can be easily identified. Using the original master plate, these recombinant clones are picked up and subcultured using the same procedure to obtain a pure recombinant clone.

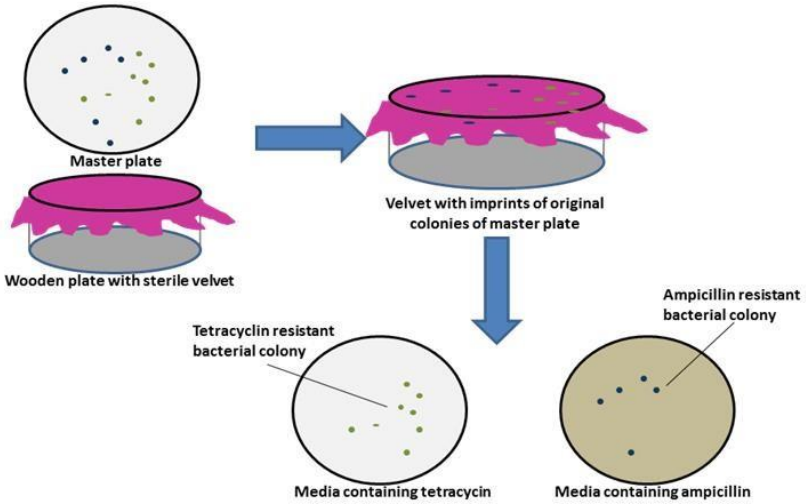


Fig 2.2.8.1 : Selection through recombinant bacteria by replica plating

Screening for expression:

Gene expression involves the synthesis of mRNA through transcription followed by synthesis of protein through translation. If the purpose of cloning is to express a foreign gene, it is necessary to check the expression both at the mRNA and protein level in terms of quality and quantity.

Screening for foreign gene mRNA transcript can be done by:

Northern blotting: It involves the electrophoresis for the separation of RNA on the basis of size, and then transfer of RNA from the electrophoresis gel to the blotting membrane. It is then detected by a hybridization probe complementary to target sequence.

Reverse transcriptase PCR: The RNA strand is reverse transcribed into its DNA complement (cDNA) using the enzyme reverse transcriptase and the resulting cDNA are then amplified using routine PCR method.

Screening for foreign protein is done by:

Western blotting: It involves the process of electrophoretic separation of cellular proteins followed by transfer to a blotting membrane, incubation with a complimentary primary antibody probe and detection with a labelled secondary antibody.

Activity/functional testing assay or staining: The proteins which have specific functionality or activity can be tested *in-vitro* to confirm the presence of the same. For example, protease activity testing or staining.

Quantitative evaluation of the expression levels of the protein is necessary to achieve desired amount of protein.

1. c-DNA libraries and Genomic libraries c-
DNA libraries and Genomic libraries

In higher eukaryotes, gene expression is tissue-specific. Only certain cell types show moderate to high expression of a single gene or a group of genes. For example, the genes encoding globin proteins are expressed only in erythrocyte precursor cells, called reticulocytes. Using this information a target gene can be cloned by isolating the mRNA from a specific tissue. The specific DNA sequences are synthesized as copies from mRNAs of a particular cell type, and cloned into bacteriophage vectors. cDNA (complementary DNA) is produced from a fully transcribed mRNA which contains only the expressed genes of an organism. Clones of such DNA copies of mRNAs are called cDNA clones. A cDNA library is a combination of cloned cDNA fragments constituting some portion of the transcriptome of an organism which are inserted into a number of host cells. In eukaryotic cells, the mRNA is spliced before translation into protein. The DNA synthesized from the spliced mRNA doesn't have introns or non-coding regions of the gene. As a result, the protein under expression can be sequenced from the DNA which is the main advantage of cDNA cloning over genomic DNA cloning.

The construction of cDNA library involves following steps-

1. Isolation of mRNA
2. Synthesis of first and second strand of cDNA
3. Incorporation of cDNA into a vector
4. Cloning of cDNAs.

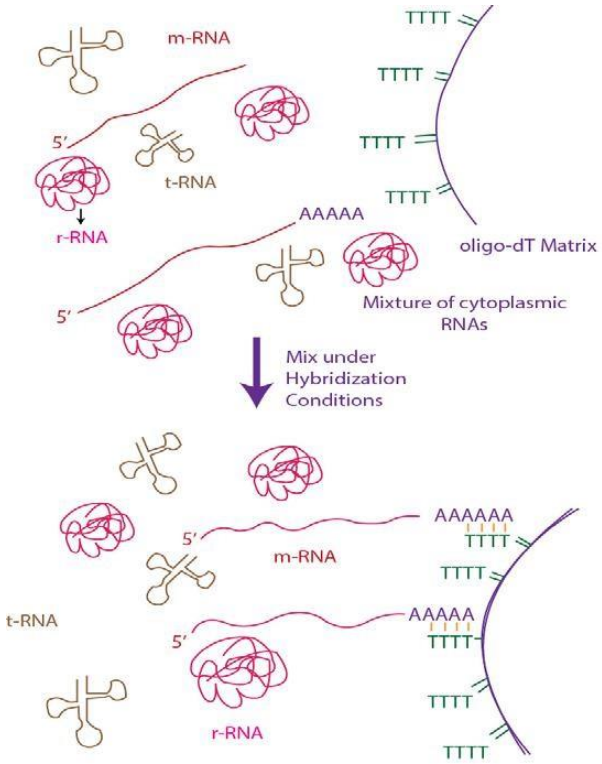
1. Isolation of mRNA

It involves the isolation of total mRNA from a cell type or tissue of interest.
The amount of desired mRNA can be increased by following ways-

- Chromatographic purification of mRNA using oligo-dT column, which retains mRNA molecules, resulting in their enrichment.
- Spinning down mRNA by density gradient centrifugation.
- mRNA preparation from specialized cell types, e.g. developing seeds, chicken oviduct, erythrocytes, β cells of pancreas etc.

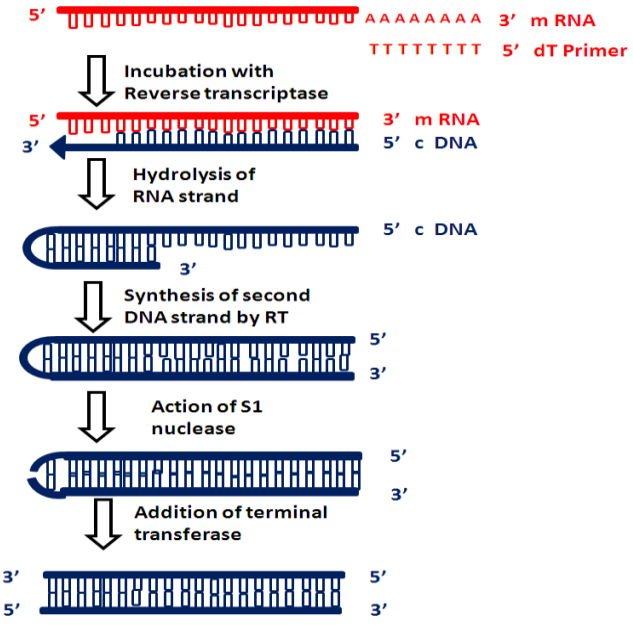
The 3′ ends of eukaryotic mRNA consist of a string of 50 – 250 adenylate residues (poly A Tail) which makes the separation easy from the much more prevalent rRNAs and tRNAs in a cell extract using a column containing oligo-dTs tagged onto its matrix.

When a cell extract is passed through an oligo-dT column, the mRNAs bind to the column due to the complementary base-pairing between poly (A) tail and oligo-dT. Other RNAs (ribosomal RNAs and transfer RNAs) flow through as unbound fraction. The bound mRNAs can then be eluted using a low-salt buffer.



2. Synthesis of first and second strand of cDNA

- mRNA being single-stranded cannot be cloned as such and is not a substrate for DNA ligase. It is first converted into DNA before insertion into a suitable vector which can be achieved using reverse transcriptase (RNA-dependent DNA polymerase or RTase) obtained from avian myeloblastosis virus (AMV).
- A short oligo (dT) primer is annealed to the Poly (A) tail on the mRNA.
- Reverse transcriptase extends the 3′-end of the primer using mRNA molecule as a template producing a cDNA: mRNA hybrid.
-



Synthesis of first and second strand of cDNA.

The mRNA from the cDNA: mRNA hybrid can be removed by RNase H or Alkaline hydrolysis to give a ss-cDNA molecule.

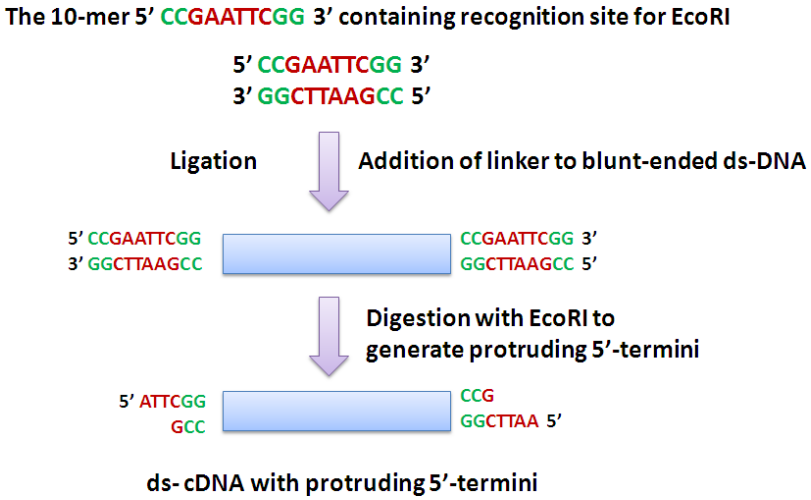
- No primer is required as the 3´end of this ss-cDNA serves as its own primer generating a short hairpin loop at this end. This free 3´-OH is required for the synthesis of its complementary strand.
- The single stranded (ss) cDNA is then converted into double stranded (ds) cDNA by either RTase or *E. coli* DNA polymerase.
- The ds-cDNA can be trimmed with S1 nuclease to obtain blunt–ended ds-cDNA molecule followed by addition of terminal transferase to tail the cDNA with C's and ligation into a vector.

3. Incorporation of cDNA into a vector

The blunt-ended cDNA termini are modified in order to ligate into a vector to prepare ds-cDNA for cloning. Since blunt-end ligation is inefficient, short restriction-site linkers are first ligated to both ends.

Linker

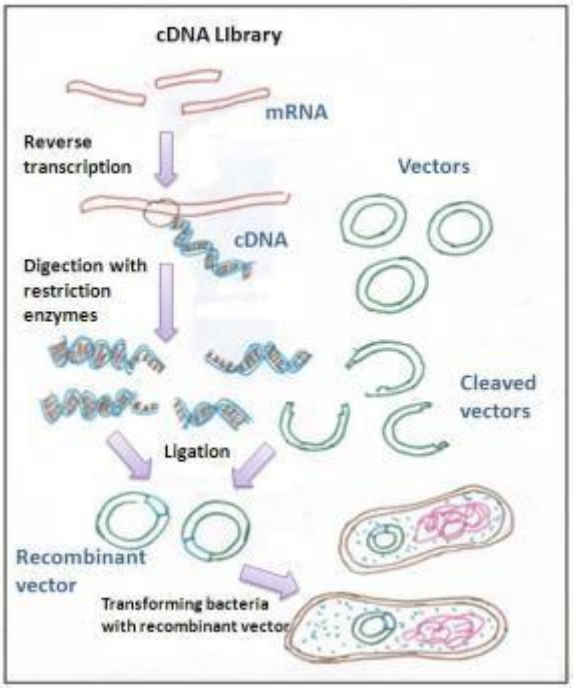
It is a double-stranded DNA segment with a recognition site for a particular restriction enzyme. It is 10-12 base pairs long prepared by hybridizing chemically synthesized complementary oligonucleotides. The blunt ended ds-DNAs are ligated with the linkers by the DNA ligase from T4 Bacteriophage.



Modification of cDNA termini using linkers

The resulting double-stranded cDNAs with linkers at both ends are treated with a restriction enzyme specific for the linker generating cDNA molecules with sticky ends. Problems arise, when cDNA itself has a site for the restriction enzyme cleaving the linkers. This can be overcome using an appropriate modification enzyme (methylase) to protect any internal recognition site from digestion which methylates specific bases within the restriction-site sequence, thereby, preventing the restriction enzyme binding.

Ligation of the digested ds-cDNA into a vector is the final step in the construction of a cDNA library. The vectors (e.g. plasmid or bacteriophage) should be restricted with the same restriction enzyme used for linkers. The *E. coli* cells are transformed with the recombinant vector, producing a library of plasmid or λ clones. These clones contain cDNA corresponding to a particular mRNA



4. Cloning of cDNAs

cDNAs are usually cloned in phage insertion vectors. Bacteriophage vectors offer the following advantageous over plasmid vectors,

- are more suitable when a large number of recombinants are required for cloning low-abundant mRNAs as recombinant phages are produced by *in vitro* packaging.

can easily store and handle large numbers of phage clones, as compared to the bacterial colonies carrying plasmids.

Plasmid vectors are used extensively for cDNA cloning, particularly in the isolation of the desired cDNA sequence involving the screening of a relatively small number of clones.

Applications of cDNA libraries/cloning

- Discovery of novel genes.
- *in vitro* study of gene function by cloning full-length cDNA.
- Determination of alternative splicing in various cell types/tissues.
- They are commonly used for the removal of various non-coding regions from the library.
- Expression of eukaryotic genes in prokaryotes as they lack introns in their DNA and therefore do not have any enzymes to cut it out in transcription process. Gene expression required either for the detection of the clone or the polypeptide product may be the primary objective of cloning.

4-3.7. Disadvantages of cDNA libraries

- cDNA libraries contain only the parts of genes found in mature mRNA. However, the sequences before and after the gene, for example, those involved in the regulation of gene expression, will not occur in a cDNA library.
- Construction of a cDNA library cannot be used for isolating the genes expressed at low levels as there will be very little mRNA for it in any cell type and may completely be out manoeuvred by the more abundant species.

GENOMIC LIBRARY

A genomic library is an organism specific collection of DNA covering the entire genome of an organism. It contains all DNA sequences such as expressed genes, non-expressed genes, exons and introns, promoter and terminator regions and intervening DNA sequences.

4-4.2. Construction of genomic library

Construction of a genomic DNA library involves isolation, purification and fragmentation of genomic DNA followed by cloning of the fragmented DNA using suitable vectors. The eukaryotic cell nuclei are purified by digestion with protease and organic (phenol-chloroform) extraction. The derived genomic DNA is too large to incorporate into a vector and needs to be broken up into desirable fragment sizes. Fragmentation of DNA can be achieved by physical method and enzymatic method. The library created contains representative copies of all DNA fragments present within the genome.

4-4.2.1. Mechanisms for cleaving DNA

(a) Physical method

It involves mechanical shearing of genomic DNA using a narrow-gauge syringe needle or sonication to break up the DNA into suitable size fragments that can be cloned. Typically, an average DNA fragment size of about 20 kb is desirable for cloning into λ based vectors. DNA fragmentation is random which may result in variable sized DNA fragments. This method requires large quantities of DNA.

(b) Enzymatic method

- It involves use of restriction enzyme for the fragmentation of purified DNA.

This method is limited by distribution probability of site prone to the action of restriction enzymes which will generate shorter DNA fragments than the desired size.

- If, a gene to be cloned contains multiple recognition sites for a particular restriction enzyme, the complete digestion will generate fragments that are generally too small to clone. As a consequence, the gene may not be represented within a library.
- To overcome this problem, partial digestion of the DNA molecule is usually carried out using known quantity of restriction enzyme to obtain fragments of ideal size.
- The two factors which govern the selection of the restriction enzymes are- type of ends (blunt or sticky) generated by the enzyme action and susceptibility of the enzyme to chemical modification of bases like methylation which can inhibit the enzyme activity.
- The fragments of desired size can be recovered by either agarose gel electrophoresis or sucrose gradient technique and ligated to suitable vectors.

The complete (a) and partial (b) digestion of a DNA fragment using restriction enzymes.

Partial restriction digestion is achieved using restriction enzymes that produce blunt or sticky ends as described below

i. Restriction enzymes generating blunt ends

The genomic DNA can be digested using restriction enzymes that generate blunt ends e.g. *HaeIII* and *AluI.*

```
HaeIII: 5'-GG|CC-3'   AluI: 5'-AG|CT-3'
        3'-CC|GG-5'         3'-TC|GA-5'
```

Blunt ends are converted into sticky ends prior to cloning. These blunt ended DNA fragments can be ligated to oligonucleotides that contain the recognition sequence for a restriction enzyme called linkers or possess an overhanging sticky end for cloning into particular restriction sites called adaptors.

Linkers

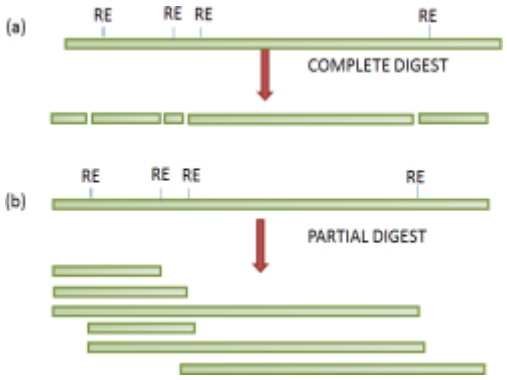
Linkers are short stretches of double stranded DNA of length 8-14 bp that have recognition site for restriction enzymes. Linkers are ligated to blunt end DNA by ligase enzyme. The linker ligation is more efficient as compared to blunt-end ligation of larger molecules because of the presence of high concentration of these small molecules in the reaction. The ligated DNA can be digested with appropriate restriction enzyme generating cohesive ends required for cloning in a vector. The restriction sites for the enzyme used to generate cohesive ends may be present within the target DNA fragment which may limit their use for cloning.

Adapters

These are short stretches of oligonucleotide with cohesive ends or a linker digested with restriction enzymes prior to ligation. Addition of adaptors to the ends of a DNA converts the blunt ends into cohesive ends.

ii. Restriction enzymes that generate sticky ends

Genomic DNA can be digested with commonly available restriction enzymes that generate sticky ends. For example, digestion of genomic DNA with the restriction enzyme *Sau3AI* (recognition sequence 5'-GATC-3') generates DNA fragments that are compatible with the sticky end produced by *BamHI* (recognition sequence 5'-GGATCC-3') cleavage of a vector. Once the DNA fragments are produced, they are cloned into a suitable vector.



Construction of genomic library

Cloning of genomic DNA

Various vectors are available for cloning large DNA fragments. λ phage, yeast artificial chromosome, bacterial artificial chromosome etc. are considered as suitable vectors for larger DNA and λ replacement vectors like *λDASH* and *EMBL3* are preferred for construction of genomic DNA library. T4 DNA ligase is used to ligate the selected DNA sequence into the vector.

(1) λ replacement vectors

The *λEMBL* series of vectors are widely used for genomic library construction. The multiple cloning sites of these vectors flanking the stuffer fragment contain opposed promoters for the T3 and T7 RNA polymerases. The restriction digestion of the recombinant vector generates short fragments of insert DNA left attached to these promoters. This generates RNA probes for the ends of the DNA insert. These vectors can be made conveniently, directly from the vector, without recourse to sub-cloning.

(2) High-capacity vectors

The high capacity cloning vectors used for the construction of genomic libraries are cosmids, bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs) and yeast artificial chromosomes (YACs). They are designed to handle longer DNA inserts, much larger than for λ replacement vectors. So they require lower number of recombinantsto be screened for identification of a particular gene of interest.

The recombinant vectors and insert combinations are grown in *E. coli* such that a single bacterial colony or viral plaque arises from the ligation of a single genomic DNA fragment into the vector.

Number of clones required for a library

The number of clones to be pooled depends upon the size of the genome *f* and average size of the cloned DNA.

Let (*f*) be the fraction of the genome size compared to the average individual cloned fragment size, would represent the lowest possible number of clones that the library must contain.

The minimum number of clones required can be calculated as-

***f*= genome size/ fragment size**

For the *E. coli* genome (4.6 Mb) with an average cloned fragment size of 5 kb, *f* will be 920.

The number of independent recombinants required in the library must be greater than *f*, as sampling variation leads to the several times inclusion and exclusion of some sequences in a library of just *f* recombinants. In 1976, Clarke and Carbon derived a formula to calculate probability (P) of including any DNA sequence in a random library of N independent recombinants.
The actual number of clones required can be calculated as-

$$N = \frac{\text{In } (1-P)}{\text{In } (1-1/f)}$$

where N= number of clones and P= probability that a given gene will be present.
Bigger the library better will be the chance of finding the gene of interest. The pooling together of either recombinant plaques or bacterial colonies generates a primary library.

Amplified library

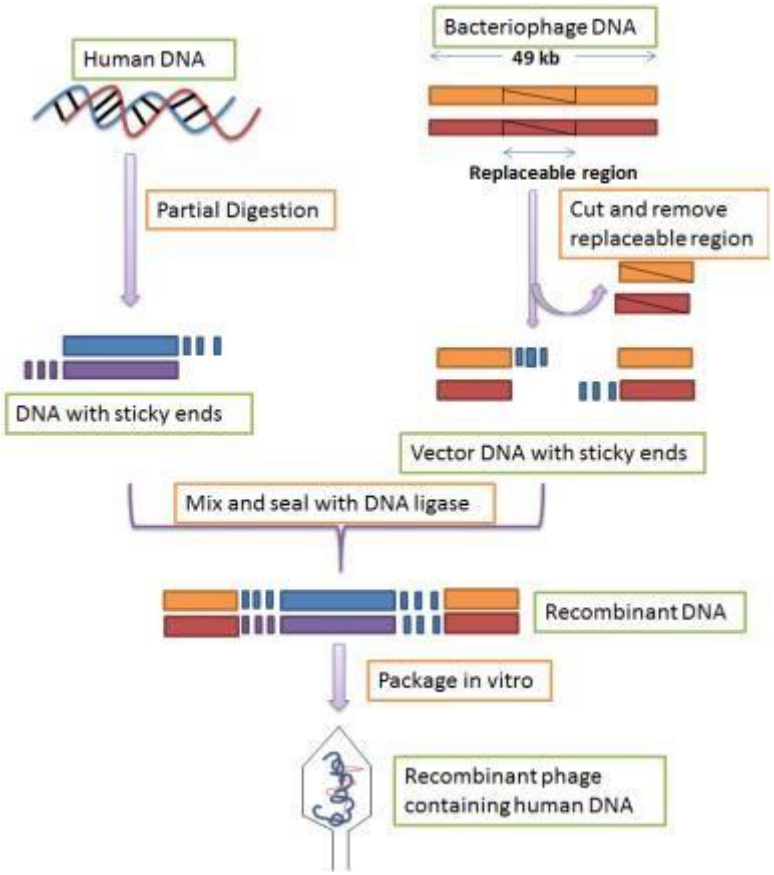
- The primary library created is usually of a low titer and unstable. The stability and titer can be increased by amplification. For this, the phages or bacterial colonies are plated out several times and the resulting progenies are collected to form an amplified library.
- The amplified library can then be stored almost indefinitely due to longshelf-life of phages.
- It usually has a much larger volume than the primary library, and consequently may be screened several times.

It is possible that the amplification process will result in the composition of the amplified library not truly reflecting the primary one.

- Certain DNA sequences may be relatively toxic to *E. coli* cells. As a consequence bacteria harboring such clones will grow more slowly than other bacteria harboring non-toxic DNA sequences. Such problematic DNA sequences present in the primary library may be lost or under-represented after the growth phase required to produce the amplified library.

Subgenomic library

Subgenomic library is a library which represents only a fraction of the genome. Enhancing the fold of purification of target DNA is crucial for subgenomic DNA libraries which can be achieved by multiple, sequential digestion when information of the restriction map of the sequences of interest is known. After initial purification of a given fragment, the purification can further be increased by redigestion with another enzyme generating a smaller (clonable) fragment relative to original DNA.



Advantages of genomic libraries

- Identification of a clone encoding a particular gene of interest.
- It is useful for prokaryotic organisms having relatively small genomes.
- Genomic libraries from eukaryotic organisms are very important to study the genome sequence of a particular gene, including its regulatory sequences and its pattern of introns and exons.

Disadvantages of genomic library

- Genome libraries from eukaryotes having very large genomes contain a lot of DNA which does not code for proteins and also contain non-coding DNA such as repetitive DNA and regulatory regions which makes them less than ideal.
- Genomic library from a eukaryotic organism will not work if the screening method requires the expression of a gene.

Applications

- To determine the complete genome sequence of a given organism.
- To study and generate transgenic animals through genetic engineering, serving as a source of genomic sequence.
- To study the function of regulatory sequences *in vitro.*
- To study the genetic mutations.
- Used for genome mapping, sequencing and the assembly of clone contigs

3. TRANSGENESIS

Transgenesis is the process of introducing an exogenous gene — called a transgene — into a living organism so that the organism will exhibit a new property and transmit that property to its offspring. Transgenesis can be facilitated by liposomes, plasmid vectors, viral vectors, pronuclear injection, protoplast fusion, and ballistic DNA injection.

Transgenic organisms are able to express foreign genes because the genetic code is similar for all organisms. This means that a specific DNA sequence will code for the same protein in all organisms. Due to this similarity in protein sequence, scientists can cut DNA at these common protein points and add other genes. An example of this is the "super mice" of the 1980s. These mice were able to produce the human protein tPA to treat blood clots.

The most common type of transgenesis research is done with bacteria and viruses which are able to replicate foreign DNA. The plasmid DNA is cut using restriction enzymes, while the DNA to be copied is also cut with the same restriction enzyme, producing complementary sticky-ends. This allows the foreign DNA to hybridise with the plasmid DNA and be sealed by DNA ligase enzyme, creating a genetic code not normally found in nature. Altered DNA is inserted into plasmids for replication.

Transgenic Animal

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to the gene itself, the DNA usually includes other sequences to enable it

- to be incorporated into the DNA of the host and
- to be expressed correctly by the cells of the host.
- Transgenic sheep and goats have been produced that express foreign proteins in their milk.
- Transgenic chickens are now able to synthesize human proteins in the "white" of their eggs.

These animals should eventually prove to be valuable sources of proteins for human therapy.

Transgenic cattle

Transgenic cows are genetically modified (GM) cows. They have an extra gene or genes inserted into their DNA. The extra gene may come from the same species or from a different species.

The extra gene (transgene) is present in every cell in the transgenic cow. However, it's only expressed in mammary tissue. This means that the transgene's protein will only be found in the cow's milk and can only be extracted from there. Since 2000, scientists at AgResearch have been successfully producing transgenic cows that make modified milk or produce therapeutic proteins to treat human diseases.

Transgenic Pig

Transgenic pigs have also been produced by fertilizing normal eggs with sperm cells that have incorporated foreign DNA. This procedure, called sperm-mediated gene transfer

(SMGT) may someday be able to produce transgenic pigs that can serve as a source of transplanted organs for humans

Transgenic Sheep

Until recently, the transgenes introduced into sheep inserted randomly in the genome and often worked poorly. However, in July 2000, success at inserting a transgene into a specific gene locus was reported. The gene was the human gene for **alpha1-antitrypsin**, and two of the animals expressed large quantities of the human protein in their milk.

Transgenic Fish

Genetically modified fish (**GM fish**) are organisms from the taxonomic clade which includes the classes Agnatha (jawless fish), Chondrichthyes (cartilaginous fish) and Osteichthyes (bony fish) whose genetic material (DNA) has been altered using genetic engineering techniques. In most cases, the aim is to introduce a new trait to the fish which does not occur naturally in the species, i.e. transgenesis.

GM fish are used in scientific research and kept as pets. They are being developed as environmental pollutant sentinels and for use in aquaculture food production. In 2015, the Aqu Advantage salmon was approved by the US Food and Drug Administration (FDA) for commercial production, sale and consumption, making it the first genetically modified animal to be approved for human consumption. Some GM fish that have been created have promoters driving an over-production of "all fish" growth hormone. This results in dramatic growth enhancement in several species, including salmonids, carps and tilapias.

Critics have objected to GM fish on several grounds, including ecological concerns, animal welfare concerns and with respect to whether using them as food is safe and whether GM fish are needed to help address the world's food needs.

Cloning and cloned animals (Dolly sheep)

Many people first heard of cloning when Dolly the Sheep showed up on the scene in 1997. Artificial cloning technologies have been around for much longer than Dolly, though.

There are two ways to make an exact genetic copy of an organism in a lab: artificial embryo twinning and somatic cell nuclear transfer.

1. Artificial Embryo Twinning

Artificial embryo twinning is a relatively low-tech way to make clones. As the name suggests, this technique mimics the natural process that creates identical twins.

In nature, twins form very early in development when the embryo splits in two. Twinning happens in the first days after egg and sperm join, while the embryo is made of just a small number of unspecialized cells. Each half of the embryo continues dividing on its own, ultimately developing into separate, complete individuals. Since they developed from the same fertilized egg, the resulting individuals are genetically identical.

Artificial embryo twinning uses the same approach, but it is carried out in a Petri dish instead of inside the mother. A very early embryo is separated into individual cells, which are allowed to divide and develop for a short time in the Petri dish. The embryos are then placed into a surrogate mother, where they finish developing. Again, since all the embryos came from the same fertilized egg, they are genetically identical.

2. Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT), also called nuclear transfer, uses a different approach than artificial embryo twinning, but it produces the same result: an exact genetic copy, or clone, of an individual. This was the method used to create Dolly the Sheep.

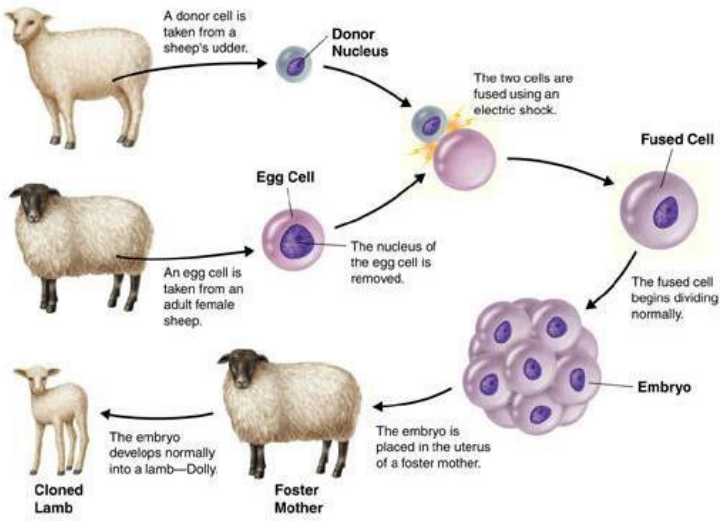
Somatic cell: A somatic cell is any cell in the body other than sperm and egg, the two types of reproductive cells. Reproductive cells are also called germ cells. In mammals, every somatic cell has two complete sets of chromosomes, whereas the germ cells have only one complete set.

Nuclear: The nucleus is a compartment that holds the cell's DNA. The DNA is divided into packages called chromosomes, and it contains all the information needed to form an organism. It's small differences in our DNA that make each of us unique.

Transfer: Moving an object from one place to another. To make Dolly, researchers isolated a **somatic cell** from an adult female sheep. Next they removed the nucleus and all of its DNA from an egg cell. Then they **transferred** the **nucleus**from the somatic cell to the egg cell. After a couple of chemical tweaks, the egg cell, with its new nucleus, was behaving just like a freshly fertilized egg. It developed into an embryo, which was implanted into a surrogate mother and carried to term. (The transfer step is most often done using an electrical current to fuse the membranes of the egg and the somatic cell.)

The lamb, Dolly, was an exact genetic replica of the adult female sheep that donated the somatic cell. She was the first-ever mammal to be cloned from an adult somatic cell.

Cloning Dolly the sheep



Dolly the sheep, as the first mammal to be cloned from an adult cell, is by far the world's most famous clone. However, cloning has existed in nature since the dawn of life. From asexual bacteriato 'virgin births' in aphids, clones are all around us and are fundamentally no different to other organisms. A clone has the same DNA sequence as its parent and so they are genetically identical.

Several clones had been produced in the lab before Dolly, including frogs, mice, and cows, which had all been cloned from the DNA from embryos. Dolly was remarkable in being the first mammal to be cloned from an adult cell. This was a major scientific achievement as it

demonstrated that the DNA from adult cells, despite having specialised as one particular type of cell, can be used to create an entire organism.

Animal cloning from an adult cell is much more difficult than from an embryonic cell. So, when scientists working at the Roslin Institute in Scotland produced Dolly, the only lamb born from 277 attempts, it was a major news story around the world.

To produce Dolly, scientists used an udder cell from a six-year-old Finn Dorset white sheep. They had to find a way to 'reprogram' the udder cells - to keep them alive but stop them growing – which they achieved by altering the growth medium (the 'soup' in which the cells were kept alive). Then they injected the cell into an unfertilized egg cell which had had its nucleus removed, and made the cells fuse by using electrical pulses. The unfertilized egg cell came from a Scottish Blackface ewe. When the research team had managed to fuse the nucleus from the adult white sheep cell with the egg cell from the black-faced sheep, they needed to make sure that the resulting cell would develop into an embryo. They cultured it for six or seven days to see if it divided and developed normally, before implanting it into a surrogate mother, another Scottish Blackface ewe. Dolly had a white face.

From 277 cell fusions, 29 early embryos developed and were implanted into 13 surrogate mothers. But only one pregnancy went to full term, and the 6.6 kg Finn Dorset lamb 6LLS (alias Dolly) was born after 148 days.

DNA fingerprinting

DNA fingerprinting, also called DNA typing, DNA profiling, genetic fingerprinting, genotyping, or identity testing, in genetics, method of isolating and identifying variable elements within the base-pair sequence of DNA (deoxyribonucleic acid).

The technique was developed in 1984 by British geneticist Alec Jeffreys, after he noticed that certain sequences of highly variable DNA (known as minisatellites), which do not contribute to the functions of genes, are repeated within genes. Jeffreys recognized that each individual has a unique pattern of minisatellites (the only exceptions being multiple individuals from a single zygote, such as identical twins).

1. The first step is to obtain DNA sample of the individual (blood, hair root, Semen, etc.
2. DNA is also isolated from bloodstains, semen stains or hair root from the body of the victim or from victim's cloth even after many hours of any criminal offence. Even it can be obtained from vaginal swabs of rape victims. The amount of DNA needed for developing fingerprints is very small, only a few nanograms.
3. The DNA is digested with a suitable restriction endonuclease enzyme, which cuts them into fragments.
4. The fragments are subjected to gel electrophoresis by which the fragments are separated according to their size.
5. The separated fragments are copied onto a nitrocellulose filter membrane by Southern blotting technique.
6. Special DNA probes are prepared in the laboratory and made radioactive by labeling with radioactive isotopes.
7. These probes contain repeated sequences of bases complimentary to those on mini satellites.

8. The DNA on the nitrocellulose filter membrane is hybridized with the radioactive probes and the free probes are washed off.
9. The bands to which the radioactive probes have been hybridized are detected through autoradiography. This is a technique where an X-ray film is exposed to the nitrocellulose membrane to mark the places where the radioactive DNA probes have bound to the DNA fragments. These places are marked as dark bands when X-ray film is exposed.
10. The dark bands on the X-ray film represent the DNA fingerprints or DNA profiles.
11. Comparison is made between the banding pattern of collected DNA sample and suspected human subject to confirm the criminal with hundred percent accuracy.

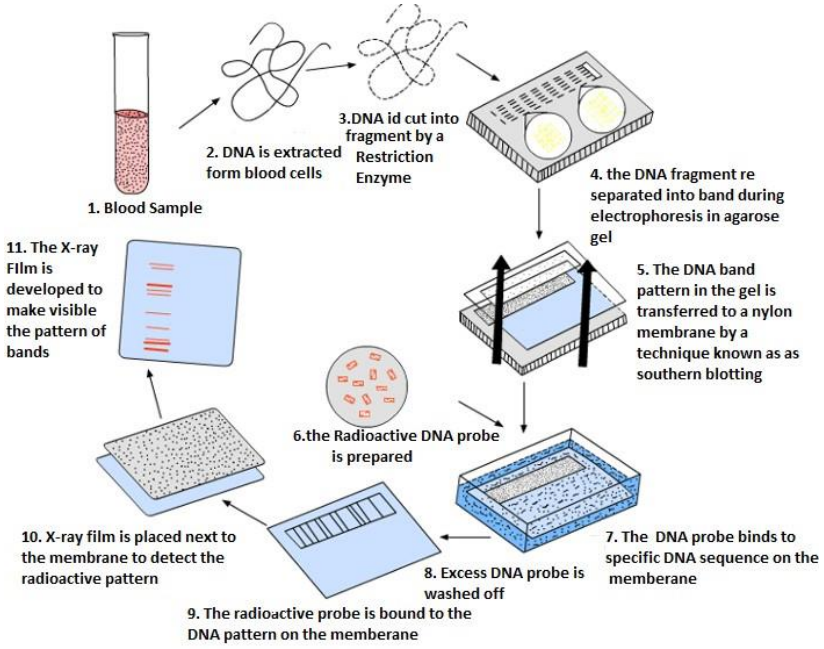


Fig. Steps in DNA fingerprinting technique

Significance:

1. The technique is extensively used as confirmatory test in crime detection in cases of rape and murder.
2. Disputed parentage can be solved by the technique.
3. This method can confirm species of more closeness or far apart from evolutionary point of view so that taxonomical problems can be solved.
4. The technique also can be used to study the breeding pattern of endangered animals.
5. Clinically this method can be used in restoring the health of blood cancer patients.

UNIT- III

Organic Evolution

The term evolution (L. evolution = unfolding or unrolling) means a gradual orderly change from one condition to another. The term evolution was coined by Herbert Spencer.

The evolution may briefly be stated as "Descent with modifications" it means that the living beings modify and adapt according to the ever changing environmental needs. These modifications keep accumulating in the organism's generations after generations, resulting in more complex and better adapted new species. Darwin has described evolution as "Descent with modification".

Basic Concept of Organic Evolution

Concept of organic evolution envisages 'Continuity of life with constant modification'. It suggests that-

- Environmental conditions in nature are ever changing.
- Organisms have an inherent character of changing to the changing environmental conditions. This is called adaptability or adaptation.
- Such adaptive changes in organisms lead to the 'Origin of new species' (Evolution).
- Since changes in the organisms are due to adaptations, new species are always better adapted and more organized than their ancestors.
- Different members of a species, on being adapted to different environments, diversify and evolve along several divergent lines.
- All the presents' day species had a common ancestor at some or other tome of their evolution (Monophyletic Geneology).
- Individuals migrate from their place of origin to varied geographical areas and generally adapt to different environmental conditions. This results in the formation of several new species from one ancestral species (Divergent Evolution).
- Evolution is a very complex and extremely slow process. It is not possible to see one type of animals changing to other, but presence of Intergrading organisms supports the concept of evolution.

Theories of Organic Evolution

I. Lamarck's Theory

Jean Baptiste de Lamarck (1774 -1829)

The great French evolutionist, Jean Baptiste de Lamarck is considered as the founder of the theory of descent.

Jean Baptiste de Lamarck: Jean Baptiste de Lamarck (1774 -1829), a French biologist is well known for his concept, inheritance of acquired characters in organisms for the formation of new species, which he explained in his book *Philosophic Zoologique* in 1809. His theory is based upon the following four laws or propositions.

1. The internal forces of life trend to increase the size of the organism and its body parts.
2. Due to changed conditions, necessities and new wants, new organs are formed in the body, or pre-existing organs are modified.
3. Development of an organ is in direct proportion to its employment.
4. New characters which result due to changed environment or needs and necessities (i.e. acquired characters) are transmitted to the offspring. In the course of large number of generations, the species is modified into a new one.

With the help of these four laws Lamarck explained the mechanism of evolution in the following manner. According to him, the changes of environment; soil, food, temperature etc. act directly upon plants and indirectly upon animals and man.

Due to the changed conditions of life, animals are subjected to new surroundings resulting in new wants or needs. To fulfill these needs new organs are formed or the pre-existing organs are modified. For example, certain changes in the environment in the geological past brought about very prominent changes in Giraffe, snakes and water birds. When the ancestors of Giraffe did not find any herbage to graze upon, they were forced to graze on leaves of taller trees.

This involved stretching of forelegs and neck. Thus, through a number of generations, today's long necked, tall Giraffe resulted.

Fig: 1 Long necked, tall Giraffe.

Use and Disuse of organs play an important role in the mechanism of evolution. Lamarck explained that in **Ostrich,** the legs are long and powerful and the wings are poorly developed because Ostrich uses its legs and relies on its speed of running to escape from its enemies. Similarly, modification of forelimbs into wings in case of birds is another example. Disuse of certain organs results in their reduction. In Whales, the hind limbs are lost because of disuse.

Due to overcrowding, there is competition between the organisms for food and shelter. The stronger and larger organisms destroy the weaker and smaller organism. Organisms acquire new characters which help them to succeed in this competition.

According to Lamarck, the new characters acquired, or the advantages gained by the individual as a result of the structural changes resulting through use and disuse, are transmitted to the offspring. On this account, Lamarck's theory is often referred to as the **theory of inheritance of acquired characters.**

Salient features of Lamarckism:

- He observed that

i. Species change under changing external influences,
ii. There is a fundamental unity underlying the diversity of species
iii. The species progressively develop.

- Based on these observations he assumed:

1. Living organisms and their parts tend to increase in size continuously due to internal forces of life,
2. New organs (characters) are developed in order to meet the needs of the new want and are maintained (acquired characters).
3. The development of organs and their use are proportional to the activities of these organs. Use and disuse of organs result in variations.

4. Every new character that has been acquired in the life of an individual is preserved and transmitted to the next generation by them (inheritance of acquired characters).

Thus, Lamarck proposed that variations in organisms were the result of the influence of environment.

New needs as a reaction to the environment-

Lamarck believed that environment plays an important role in influencing the form of living organisms and their external or internal organs. The influence leads to change in their habits. The change in habits results in unusual activity of an organ or structure.

For example, changes in the environment, soil, food, temperature etc, were supposed to act directly in the case of plants and indirectly in case of animals. In case of animals migration from one place to another leads to change in the environment and introduces new needs or requirement. **Lamarck** demonstrated several cases where individuals of the same species**,** grown under different environmental conditions, exhibited marked differences. He noted smaller and weaker plants in poor soil but healthy and luxuriant plants in rich soil.

The effect of needs –

Lamarck thought that change or habits may initiate the formation of a new organ or may bring the modification of the existing organ or structure.

Use and disuse –

The constant use of an organ increases its efficiency and size and leads to its better development. On the contrary if any organ is not used for a long time it leads to its degeneration. For that he explained the Giraffe example. Similarly presence of vestigial organs in animals was explained by **Lamarck** due to their continuous disuse.

Inheritance of acquired characters –

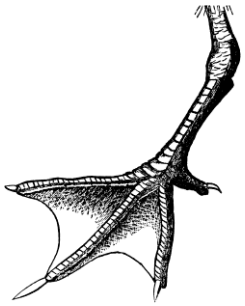
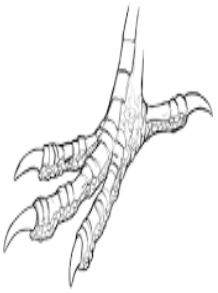
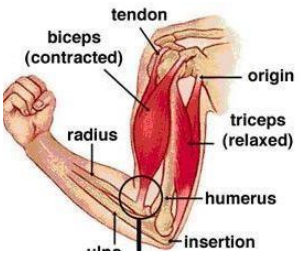
All that has been acquired by the organism during its life time due to direct or indirect environmental effects is preserved by the generation and its transmitted to the offspring's. In the offspring's these modifications become more and more pronounced if they are exposed to similar stress of the environment as was faced by their parents or ancestors. Such cumulative effects will ultimately result in the appearance of new species.

Examples

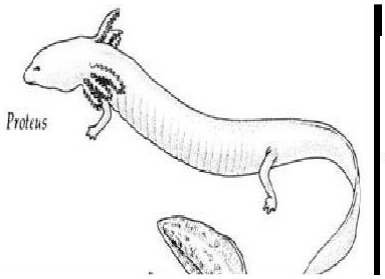
Lamarck's argument were based on direct observation of the nature. Some of the examples are explained below.

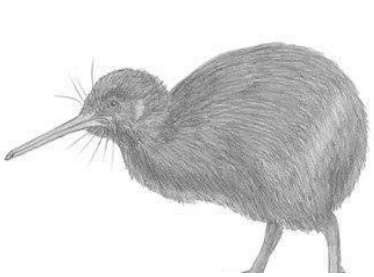
1. Ancestors of modern horse lived in soft ground in jungles. When these were replaced by dry grassy plains the horses had to graze on hard grass and to walk on dry land. These changes in habit were accompanied by changes in the molars and premolars, reduction in the number of digits and lengthening of the legs. The foot posture gradually changed to unguligrade which was suited for swift running over hard ground.

2. Giraffe obtained its long neck by stretching it upwards to reach the available food in the form of leaves from tall trees.
3. Water birds (duck etc) developed their webbed feet by constant stretching of digits and the skin between them for skimming the water surface and for swimming.
4. Clasping birds through constant perching on the twigs or branches of the trees have developed sharp and curved digits.
5. Eyes are reduced in moles because they live underground.
6. Muscles or pinna are reduced in man but are well developed and functional in rabbit, dog, elephant etc. they use their pinna to collect sound wave from the surroundings.
7. The limbs are absent in snakes, Proteus and other burrowing animals because these are of no use in crawling and burrowing and rather produced a obstacle. So these became gradually reduced and finally disappeared.
8. Biceps muscles in the right arm of blacksmith are more developed, because of its continuous use.
9. Flightless birds (Kiwi –New Zealand, Ostrich- Africa and Emu – Australia) are believed to have descended from flying birds. Kiwi , when they first reached New Zealand they were able to fly. On setting there, they did not find any need to fl, as there were no enemies on land and there was plenty of food. In due course of time, these birs are lost their ability to fly and accordingly, wings got degenerated.
10. 10.

Webbed feet of aquatic; **Birds Perching Birds Toe**; Hand showing Biceps

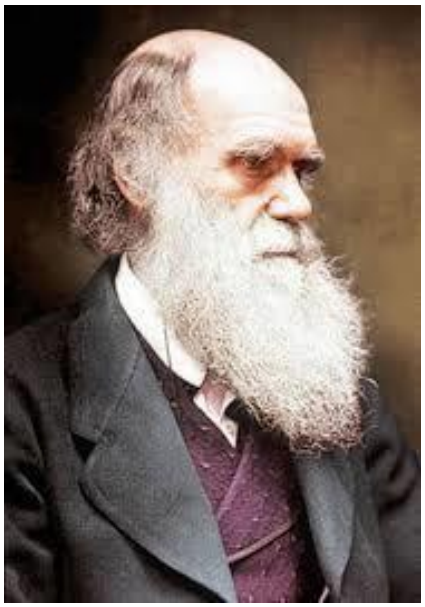




Proteus Kiwi bird

II. Darwin's Theory

Various hypotheses were suggested by scientists to explain the concept of evolution. As a result of many years of careful study and observations, **Charles Darwin** (1809-1882) proposed his theory of **natural selection** in 1859. He published a book **"The origin of Species"** in the year 1859. He proposed that the new species came about by a process called **"natural selection"**. While explaining his theory **Darwin** gave an account of any basic facts and deductions and stated how natural selection operates and results in evolution of new species.



Charles Darwin (1809-1882)

The basic facts are as follows:

1. Over production :

Every species tends to increase in a geometrical manner, provided there are no environmental checks. Lower animals produce larger number of offspring's within a short time. Drosophila, a fruit fly, completes its life cycle in 10 to 14 days. Female Drosophila produces 200 eggs at a time. If the flies formed from these eggs survive and reproduce, they may produce about 200 million individuals within a period of 40 to 50 days.

An oyster lays millions of eggs in one season. A lung fish produces 28 million eggs at a time. If all young ones survive and reproduce, the individuals resulting will be many times more than the other population. Higher animals may increase rapidly in the same manner. Darwin calculated that even a very slow breeding animal like elephant may give rise to 19 million descendents at the end of 750 years, if there is no natural check on the increase.

2. Population Stability:

Inspite of this high reproductive potential, the number of individuals in a species remains relatively constant suggesting struggle for existence.

3. Struggle for Existence:

Because of excessive breeding and resultant increase in number, there is an inevitable competition between all organisms for space, food, water, light etc. through this competition, organisms struggle for existence. This struggle takes place in three ways.

a. Intraspecific struggle.
b. Interspecific struggle.
c. Environmental struggle.

a. Intraspecific struggle takes place between members of the same species. This is often most fatal for the needs of all individuals of a species are similar and so there is severe competition among them e.g. young trees in a forest. They spring up as seedling all over, but most of them die soon for want of space and moisture or due to other causes. Some trees become tall and their branches meet one another and shut off light and air for the smaller plants growing underneath them. As a result, the smaller plants gradually perish and only large, tall trees are left in the forest. In other words, less fit ones die and taller, dominant ones continue to grow.

b. Interspecific struggle is the struggle between members of different species. This is very common because, in a community, some organisms feed on others. The plants and lower groups of animals are more commonly affected by this kind of struggle.

c. Environmental struggle is the struggle of living beings against the physical environment. It is the struggle against excess of moisture or drought, against extreme heat or cold, against lightening, earthquakes, volcanic eruptions, etc.

Thus, all these aspects of struggle are in order to procure **life conditions for survival.**

4. Genetic Variations within a Species.

Animals and plants show variations of all grades. No two individuals are exactly alike. Some of these variations are simply caused by external conditions (environmental), such as accidents, temperature, food abundance, etc. such **somatic variations,** die with the organism, and are never inherited and can have no effect on evolution.

5. Natural Selection or Survival of the Fittest.

Organisms struggle for existence. Those individuals who have favorable variations have better chances of living long enough to reproduce. They pass on their advantageous characters to the next generation. Thus, organisms with advantageous characters survive, while those which lack such variations perish. The advantageous characters are passed on to the offspring's generation after generation and the organisms become better suited for survival. Darwin described that nature selects such organisms i.e. there is **natural selection.**

The basic facts and deductions of Darwin's theory are summarized as follows

	FACTS	**CONSEQUENCE**
I	1. Rapid increase in numbers 2. Total number approximately remains same	Struggle for Existence
II	3. Struggle for Existence 4. Variations with heredity	Natural selection or Survival of the fittest
III	5. Survival of the fittest 6.Change in environment	Structural modifications leading to the origin of species.

III. Modern Synthetic Theory- Neo Darwinism

The theory of evolution as proposed by Darwin and Wallace has been modified in the light of advanced studies in Genetics, Molecular Biology, Paleontology, Ecology and Ethology (the study of behavior). This is known as Neo- Darwinism (Neo- New) or Modern Synthetic Theory. It resolves the conflict between the followers of mutation and natural selection theories. Therefore, this may be defined as "the theory of organic evolution by natural selection in inherited characteristics". The study of population genetics also forms the basis of modern views of evolutionary theory i. e. of Neo- Darwinism.

Different types of evidences support different aspects of the theory. In order to accept Neo-Darwinism evolutionary theory it is necessary to:

1. Establish the fact that evolution has taken place in the past (past evolution).
2. Show a mechanism which results in evolution (natural selection of genes).
3. Observe evolution taking place today (evolution in action).

As mentioned earlier, evidence for past evolution comes from Geology, study of age and the order of rock formation, paleontological evidence etc. Evidence of mechanism of evolution comes from mechanism of inheritance shown by Mendelian Genetics, i.e. inheritance of characters by natural selection etc. Evolution in action today is provided by studies of present population, Population Genetics (Gene pool, Genetic drift, Hardy-Weinberg principle etc.), Speciation Genetic Engineering (e.g. cultivation of Wheat and the synthesis of genes).

The important features of the modern synthetic theory of evolution are as follows:

1. Characters are determined by genes, the small units on chromosomes.
2. Changes take place in gene pool due to recombination and sudden changes in the characters i.e. Mutation. Mutations are now believed to be the raw materials for evolution by natural selection.
3. Mutations provide Variations, which enable the organism to cope with constant changes in the environment.
4. Favorable variations or adaptive characters formed either by genetic recombination or

mutations are selected by nature – i.e. natural selection because of differential reproduction. Theses adaptive characters are inherited by the organisms.

5. When such new species are isolated for a period of time, cross breeding is eliminated. i.e. there is reproductive isolation. The reproductive isolation plays an extremely important role in formation of new species.
6. The formation of new species results in evolution.
7. Neo- Darwinism or Modern Synthetic Theory can be summarized that Mutations result in variations, if they undergo natural selection under condition of isolation, results in speciation, and thus Evolution.

IV. Hugo De Vries Theory

Hugo De Vries (1848-1935)

Spontaneous Mutations form the basis of mutation theory of evolution. Hugo De Vries (1848-1935) worked on a plant *Oenothera lamarckiana*, called evening primrose. He observed 7 generations and about fifty thousand individuals of this plant.

He observed that, of these, about 800 plants showed differences of shape, flowers and leaves. De Vries described these changed varieties as mutations. When self –fertilized, they all gave rise to their own variety. Thus, according to him, variations occurred suddenly and gave rise to new species called mutants which breed their own kind.

After studying these plants carefully, Hugo De Vries proposed his theory of evolution by mutation in 1907. He held that new and distinct species are formed abruptly by virtue of mutations. De Vries explained that new species are not slowly built up through the selection of individual variations; but originated by mutations. He pointed out that each mutation is only slightly different from the parental form.

The most unique feature is the constancy with which the mutation is inherited. Many animals which Darwin recognized as varieties correspond to the elementary species of De Vries. The main difference between the natural selection theory and the mutation theory is that, the former explains that varieties arise due to selection of individual variations while the latter advocates that they arise spontaneously and at once from the original forms.

The importance of the mutation theory is as follows:

1. According to this theory, evolutionary changes lakes place in a short time.
2. The incipient stages of an organ can be explained by this theory because every stage is complete mutation. The organ may persist even when it has no value to the race.
3. The new mutations appear in large numbers and are of different kinds. So , a species cannot perish by crossing.

4. Development of unwanted or injurious characters in an organism can be explained by mutation theory. Natural selection fails to explain the occurrence of such characters.

Objections

1. Mutation was not accepted as the prime agent for evolution because, though **Oenothera** shows variety of mutations, other species did not show occurrence of such mutations.
2. Though some mutations may cause major changes, most of them produce only slight changes.
3. Similarly, the presence of flightless birds on oceanic islands cannot be explained by this theory.
4. There is no basis for mutations in evolution. It only provides a raw material for evolution. Some other forces bring about the evolutionary changes.

UNIT-IV

1. Evidences of Organic Evolution

Over a million species of living organisms are found on the earth today. Biologists believe that these organisms have evolved as result of change with time. The evidence that led biologists to conclude that evolution has taken place is of several kinds

a. Anatomical
b. Embryological
c. Paleontological
d. Biochemical etc.

a. Anatomical Evidences

In 1760 the French naturalist **Buffon** studied the foot structure of many vertebrates. He found that although they differed in appearance and functions, they all had the same basic plan. This can only be explained by assuming that animals have arisen from previous ancestral forms by modification. Two types of evidences for evolution from comparative anatomy are **homology, analogy** and **vestigial organs.**

Thus, homologous structures indicate common ancestry among organisms. They arise from the same embryonic structures and have the same basic parts. An example of homology is the forelimbs of vertebrates (Fig. 2). If we observe the forelimbs of a horse, whale, bat, bird and man, we find that they appear to be different and have different functions. The foreleg of the horse is adapted for **running,** the paddle of the whale for **swimming,** the wings of a bird or bat for **flying** and the arm of man for **grasping** and **handling**. The bones of these forelimbs, however, show the same basic structure.

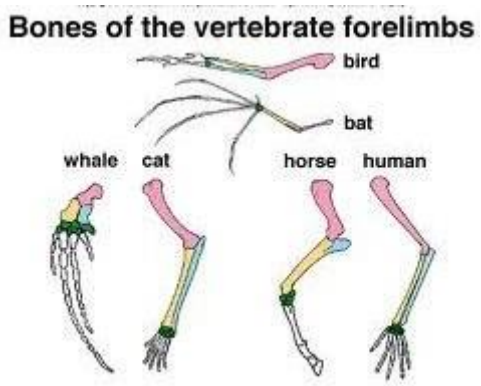


Fig. 2 Homology in the vertebrate limbs.

The brain of vertebrates (Fig. 3**)** presents another good example of homology. The brains of vertebrates ranging from fishes to mammals are constructed of similar parts: olfactory lobes, cerebral hemispheres, optic lobes, cerebellum, medulla and so on, all these parts are embedded in forebrain, midbrain and hindbrain.

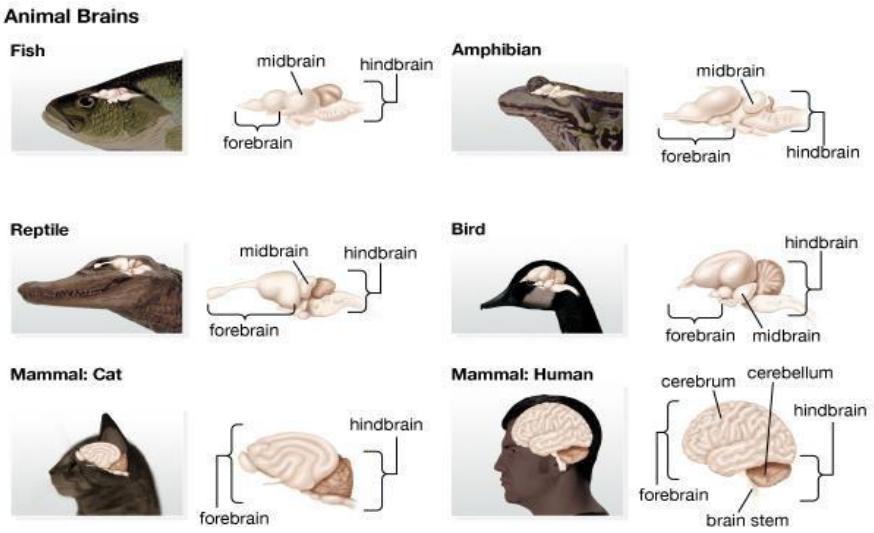


Fig. 3 Brains of vertebrates showing homology.

Analogy thus, refers to the relationship between structures which though differ anatomically but have superficial similarity due to similar functions. Often analogous organs have little gross structural resemblance to each other for example, the gills of Palaemon and lungs of man. Other analogous organs, such as the wings of insects, aves (birds) and flying mammals (bats) may have a superficial resemblance fig.4. The bones of the wings of the pterodactyle, bird and bat are homologous to each other. The insect's wing is similar to vertebrate wings in function, but it has only a superficial structural resemblance to them. It is, therefore, analogous and homoplastic.

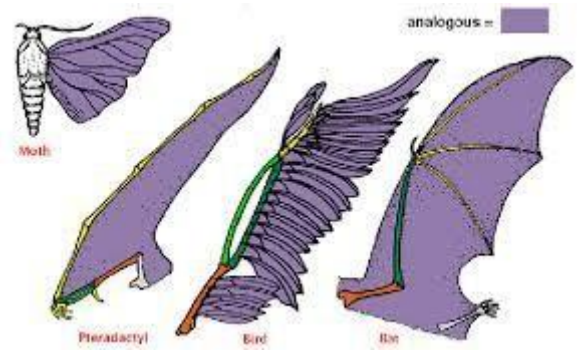


Fig. 4. Analogy and homology in wings.

Vestigial organs:

Another source of evidences stemming from the studies of comparative anatomy is the presence of organs in various animals that have no apparent functions and are useless and often small or lacking some essential part, however, in related organism, these organs remain full sized complete and functional. Such organs are called vestigial or rudimentary organs. Some common examples of vestigial organs are:

1. Muscles of the external ears

2. Panniculus carnosus
3. Coccyx
4. Vermiform appendix and caecum
5. Presence of vestigial mammary glands in male human beings.
6. Presence of vestigial nictitating membranes.
7. Presence of wisdom teeth.

b. Embryological Evidences

One of the most fascinating sources of evidence for the validity of Darwin's theory of evolution has come from the studies of comparative embryology.

Comparative embryological studies have revealed that there was one developmental pattern that could be viewed as having undergone a series of branching. All multicellular animals start their development as a single zygote and through a series of mitotic divisions, increase in cell number until a blastula is formed. The developing embryo elaborates upon the blastula stage by forming two fundamental germ layers, ectoderm and endoderm, during the course of gastrulation. After the differentiation of the ectoderm and endoderm in the gastrula, the third germ layer, mesoderm is formed. There are three distinct patterns by which developing embryo of different species produce a mesodermal layer. For instance, in annelids, molluscs and certain other invertebrates, the mesoderm develops from special cells which are differentiated early in cleavage. These cells migrate to the interior and come to lie between the ectoderm and endoderm. They then multiply to form two longitudinal cords of cells which develop into sheets of mesoderm between the ectoderm and endoderm. The mesoderm in amphibis, reptilian, birds and mammals is formed from the cells which remain associated with either ectoderm or endoderm or both and which get dissociated from them to migrate and come to lie in between both germinal layers in the form of mesoderm Fig. 5.

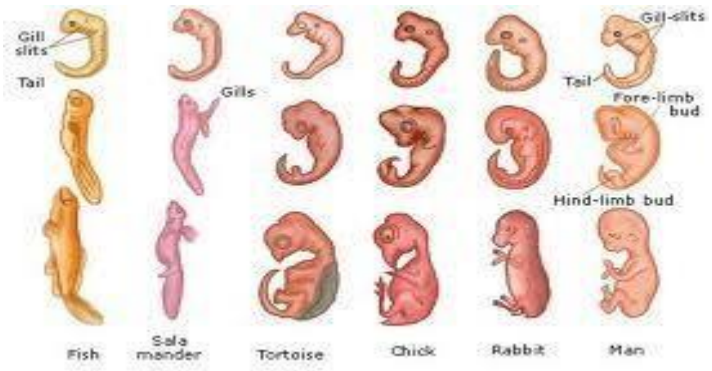


Fig. 5 Comparison of three embryonic stages from fish to man.

Despite some differences in the developmental patterns, comparative embryological studies overwhelmingly support the concept of Darwinian evolution. Another feature of embryological development that serves to link all multicellular animals is embryological source of organ systems. Regardless of the way in which the developing animals are

programmed for further differentiation of mesoderm they exhibit similar adults structures derived from the two primary germ layers. The outer covering of all multicellular animals, be it skin, scales or gelatinous material is derived from ectoderm. The universal features of ectodermally and endodermally derived tissues also indicate the presence of a common ancestral type early in the evolutionary history.

Von Baer's principles of embryonic differentiations constitute a better guide to embryological evidence for evolution. These principles are as follows.

1. General characteristics appear in the development before specialized characters.
2. From the more general, the less general and finally the specialized characters appear.
3. An animal during development departs progressive from the form of other animals.
4. Young stages of an animal do not resemble with the adults of different groups but with their embryos.

c. Paleontological Evidences

The direct evidence comes from the study of fossils is called **Paleontological Evidences.** The term fossil (Latin; *fossilum*, something dug out) refers to the petrified remains or impressions of organisms that lived in past and got preserved in the sedimentary rocks.

These includes bones, teeth, shells and other hard parts of animal or plant body, and also any impressions or imprints left by previous organisms in the soft mud or the moulds and casts of entire organism.

Paleontology is the study of past life based on fossil records. Their study reveals the existence of life in past and illustrates the course of evolution of plants and animals. Actually the layers of rocks comprising fossils can be compared to the leaves of geographical record book. Their chronological sequence illustrates the sequence of evolutionary events.

A study of the fossil record provides direct evidences. They are:

1. The older rocks contain the more primitive forms of life.
2. The younger rocks nearer the surface contain more advanced and complex forms.
3. No past forms of life are identical with present day forms.
4. A study of fossil record helps to build historical sequence of biological evolution of complex organisms from simple ancestors.

Determination of Age of Fossils and Geological Time Scale

Fossils can be arranged in chronological sequence according to their age only when their correct age is determined. The age of fossils is determined by **radioactive dating technique.** Radioactive isotope of carbon (C^{14}) is used to determine the age of up to 45,000 years old fossils, while radioactive uranium (U^{238}) or potassium (K^{40}) are used for older rocks. This dating system has been designated as **"The Clock of Rocks".**

Geologists have presented this chronological sequence of fossils or the history of evolution in the form of **'Geological Time Scale'**. The earth history has been divided into five **eras**, which are further differentiated into **periods** and periods into **epochs**.

1. Archaeozoic - Era of most ancient or primitive life.
2. Proterozoic – Era of former life.
3. Palaeozoic – Era of ancient life.
4. Mesozoic - Era of middle life or age of reptiles.
5. Coenozoic – Era of recent life or age of mammals.

These major divisions or the era are separated on the basis of geological revolutions. In these revolutions intense geological disturbances occurred changing the climate and environment, placing strong selective pressures introducing new adaptations and evolving new forms of life.

1. Distribution of fossils in different strata:

The study of distribution of animals and plants in different strata of earth crust depicts the story of evolution of living beings on earth. It has been estimated that life appeared on this earth about 2700 million years ago. The early forms which appeared in **Archaeozoic** era were all soft bodied animals, hence the fossil remains are lacking. It means records of early life are unpreserved. The fossil of **Proterozoic** era include sponges, coelenterates especially jelly fishes and corals, segmented worms, arthropods, algae and fungi. Up to Cambrian period of **Palaeozoic** era, the life was represented by invertebrates alone. The reptiles appeared in Permian, evolved and became diversified in **Mesozoic** era. Coenozoic era is the age of Mammals and the most recent one.

2. Number and nature of fossils in early rocks:

The rocks of early era (Archaeozoic and Proterozoic) contain very few fossils and those of simple marine invertebrates. This shows that life evolved in sea and early forms were simple and soft bodied.

3. Disparity between the past and present life:

Fossils from different geological strata belong to different genera. There are much different from the present day forms. Many fossils present in the upper strata of rocks resemble the present day forms or bear close resemblance to them. Moreover, the extinct animals or plants differ markedly from their living allies. e.g. the extinct fishes, amphibians and reptiles are much different from present representatives of these groups. The early man was much different from modern man. This indicates that life has gradually evolved from simpler to more complex forms.

4. Phylogenetic studies or pedigree studies:

Phylogeny is the evolutionary history of the organism. It can be traced by studying fossils in different periods and their inter-relationship. e.g. Horse.

Ancestry or phylogeny of horse shows following changes for survival in grassy places.

1. Gradual increase in limb size for fast running.
2. Reduction in the number of functional digits.

3. Development of hoof to strike hard ground.
4. Increase in the size of crown of molars and premolars for grazing grass.

Thus, study of fossils leaves no doubts that species arose from previously existing different ones.

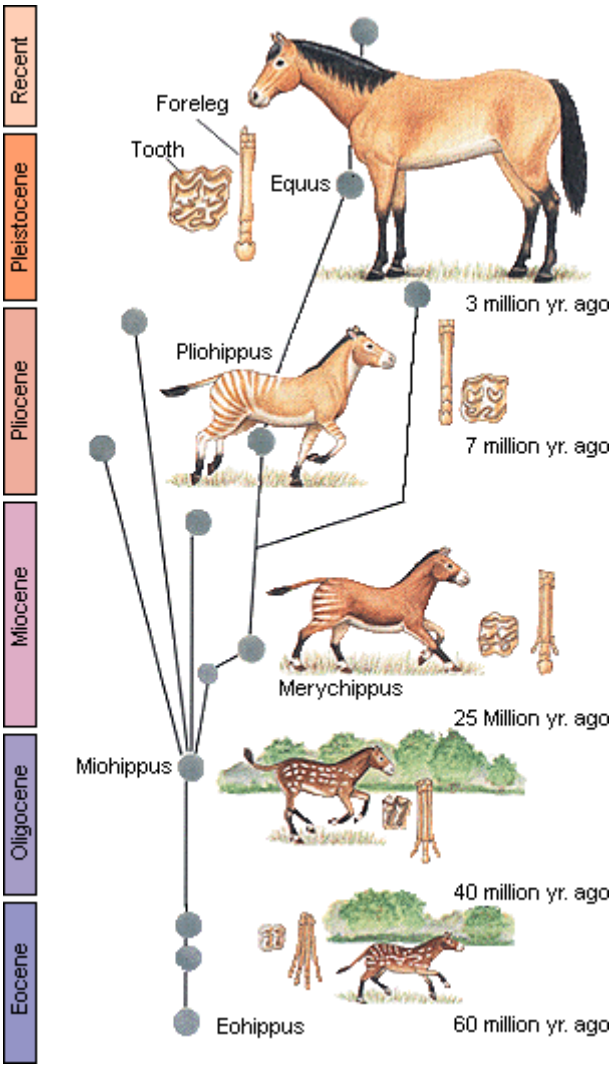


Fig.6 Phylogeny of horse to illustrate process of evolution in horse.

5 Connecting links:

The transitional fossil forms which show characteristics of two different groups of living animals are called connecting links.

Archaeopteryx:

Fossils of ***Archaeopteryx lithographica*** are obtained from upper Jurassic limestone rocks of Solenhofen in Bavaria, Germany. Its fossil is about 180 million years old. It is connecting link between reptiles and birds.

Fig.7 Archaeopteryx fossil

Reptilian characters of *Archaeopteryx:*

1. Elongated lizard like body with long pointed tail.
2. Bones solid and non pneumatic.
3. Presence of jaws with homodont dentition.
4. Vertebral centra amphicoelous or biconcave as in *Sphenodon.*
5. Cervical vertebrae fewer (9-19).
6. Presence of hand bones in reptilian pattern.
7. Tail is supported by 13-20 free caudal vertebrae.

Avian characters of *Archaeopteryx:*

1. Presence of feathers over the body.
2. Modification of forelimbs into wings.
3. Jaws are drawn into a beak.
4. Limb bones and girdle are bird like.
5. Foot consists of tars metatarsus and four clawed digits.

d. Biochemical Evidences

The most convincing evidence of common ancestry comes from the similarities in the biochemical composition, reactions and physiological activities of living beings.

a. Metabolic processes-

A remarkable similarity is noticed in the biochemical processes occurring during metabolism in all living beings from bacteria to man and in all plants and animals. For example. The process of protein synthesis, biosynthesis of various organic molecules in the body and catabolism of organic substances during respiration involves the same biochemical reactions and the same organic substances. e.g. energy in all living beings is released by the biological oxidation of glucose and is stored in ATP. The various biochemical pathways involve identical steps or reactions.

b. Enzymes-

In the above mentioned biochemical pathways the various steps are regulated by the same enzymes from bacteria to man and all plants. In all animals the same digestive enzymes are present, e.g. trypsin digests protein and amylase helps in digestion of starch.

c. Cytochrome C –

It is a respiratory pigment present in all eukaryotic cells. It forms a part of the electron transport system and in all eukaryotes accepts electrons from H^+ ions. It is formed of 104 amino acids.

In chimpanzees and humans cytochrome C molecules are identical.

d. Insulin –

Insulin analyzed from different animals (beef, sheep, pig, whale, horse, rabbit) differs only in one to three amino acid positions. Beef insulin is so similar to human insulin that it has been used for treatment of human diabetes.

e. Hormones –

In the same manner similar or identical hormones are found in large number of animal groups. **Thyroid hormone** which is found in all vertebrates is found to be identical and interchangeable.

f. Haemoglobin –

Haemoglobin is a conjugated protein. It is formed of two identical alpha chains and two identical beta chains. Each alpha chain has 141 amino acids and each beta chain has 146 amino acids. β- chain of haemoglobin of human and gorilla differ in one amino acid.

g. Blood and lymph –

The body fluids like blood, lymph and tissue fluid are similar in their composition and physiological role in all vertebrates. This indicates relationship among vertebrates. All vertebrates have haemoglobin in their RBCs, but in annelids it is dissolved plasma.

h. Blood groups –

Blood groups also help in tracing out relationship. Human beings have four blood groups A, B, AB and O. Apes are found to possess blood groups A and B but not monkeys. This indicates human beings are more closely related to Apes that to monkeys, though they have common ancestry.

i. Blood proteins

The blood proteins of various mammals are similar to a great extent, but are sharply distinguishable from other vertebrate groups.

The blood protein tests have given following informations:

1. Man is nearest to great apes, chimpanzee and gorilla and next nearest are old world monkeys, new world monkeys.
2. Dogs, cats (including lions, tigers, leopards) and bears indicate a close relationship.
3. Sheep, goats, deer and cows are closely related.
4. There is affinity between lizards, and snakes and between turtles and crocodiles.
5. The relationship have been traced out in invertebrates specially Crustacea, Insecta and Mollusca.

2. Adaptations

An adaptation is the most basic and rather self-evident concept. The organisms are surviving because they are adapted and that they are adapted because they are surviving.

The fact becomes more evident when we observe the nature and the living organisms all around. Animals like fish, lizard, birds and mammals although having markedly different organization have common feature of possessing paddle- like appendages for swimming. The flying animals all possess wing or wing like structures. It means that the organisms are adapted to their mode of life.

Therefore, adaptations can be defined as, **"Adaptation is the morphological or physiological modification in an organism to adjust itself in a particular environment".** Since the environment changed continuously, the different organisms should either change accordingly in order to survive in the rigors of the new circumstances or be ready for extinction.

Jean Baptiste deLamarck first emphasized that animals modify themselves according to the changing environment. **Darwin** believed that animals are pre adapted and seek suitable environment.

1. Aquatic adaptation

These include structural modifications that enable the organisms to live in water. These are stream- lined body, webbed-limbs, reduced neck and laterally compressed tail and gills as the respiratory organs. The deep sea forms develop phosphorescent organs. The animals living in fast running hill streams develops suckers or adhesive discs for attachment to rocks or aquatic plants or develop power of swimming against the water current. The pelagic forms develop antisinking devices. The aquatic insects and their larvae develop long feathery hairs that prevent sinking.

Aquatic adaptations develop in the animal due to their ancestors or permanently water living forms are called **primary aquatic adaptation**. If they show terrestrial ancestry then the adaptation are said to be **secondary aquatic adaptation.**

Primary aquatic adaptation

These adaptations are seen in fishes as they were evolved in water & never had terrestrial ancestry. Their features are as follows:

Body Contour: Body contour is spindle shaped with wedge shaped head, and offers little or no resistance in swimming.

Fins: Fins are the main swimming organs in fishes. The fins may or may not be paired. Paired fins are the pectoral and the pelvic whereas unpaired fins are middorsal, ventral and caudal fins.

Gills: Most fishes respire by means of the gills they develop. The gills may be external i.e. without operculum or internal i.e. with operculum.

Swim bladder or Air bladder: most of the fishes possess swim bladder that serves as a hydrostatic organ. With the help of a swim bladder a fish can float at any level in the water. In some fishes it is also associated with the production of sound.

Lateral line system:

2. Fossorial adaptation

These are changes in the organization of burrowing and cave-dwelling animals which are characterized by the possession of a slender unpigmented body with long attenuated appendages, reduced or functionless eyes, increased sense of smell and touch, changed feeding habits and ability to burrow etc.

3. Volant adaptation

The animals possessing power of flight have following structural modifications.

1. Stream-lined body
2. Pneumatic bones
3. Wings and strongly developed wing muscles
4. Reduction in the numbers of bones and other accessory structures
5. Fusion of certain bones
6. Better developed humerus and sternum, elongated radius-ulna, carpometacarpus, tarsals, metatarsals.
7. Better developed sense of smell, touch and sight etc.

4. Desert adaptation

Desert has got problems of water conservation, defense from animals and escape from the hot sand particles which might injure the sense organs. The individuals living in desert have moisture preservation capacity. They develop hygroscopic skin i.e. in lizard, water cells in the stomach wall in camel and body covering with thick dermal scales or bony scutes. The eyes, ears and nostrils are well protected against dust.

3. Hardy- Weinberg's law

The mathematical treatment of the distribution of gene and genotype frequencies in the population was developed in 1929-30, principally by **R.A. Fisher** and **J. B. Haldane** in England and **Sewall Wright** in United States, but the most fundamental idea in population genetics was offered by Englishman, **G. H. Hardy** and German, **W. Weinberg** simultaneously in the year 1908. It is known as **Hardy- Weinberg's law.** The law is the foundation of population genetics and of modern evolutionary theory.

Hardy- Weinberg demonstrated that gene frequencies are not dependent upon dominance or recessiveness. The frequencies may remain unchanged or may be conserved from generation to generation under certain conditions.

The relative frequencies of various kinds of genes in a large and randomly mating sexual panmictic population tend to remain constant from generation to generation in the absence of mutation, selection and gene flow. This is called **Hardy- Weinberg's law.**

Salient features of Hardy- Weinberg's Principle

According to Hardy- Weinberg's Principle, the gene and genotype frequencies of each allele in a population remain at an equilibrium (static) generation after generation, if that population exhibits following attributes.

1. **Random mating:** the population shall be panmictic where every gamete has an equal opportunity of fusing with any other gamete of the opposite sex. This non preferential fusion of gametes is called random mating. The natural populations may not fulfill this equality.
2. **Large population size:** The equilibrium in the gene and genotype frequencies occurs only in large sized populations. In small populations there will be significant sampling errors and there may be seen random fluctuation in the gene frequency, the so called genetic drift or random drift.
3. **Biparental mode of reproduction:** Hardy- Weinberg's principle is applicable only in biparent species.
4. **Homogenous age structure:** A population normally consists of individuals at different stages of reproductive maturity. According to Hardy- Weinberg, the population must be homogenous in this regard.
5. **Absence of evolutionary forces:** The gene frequency will remain static only in the absence of evolutionary forces like mutations, selection, genetic drift and migration.

Significance of Hardy- Weinberg's law

The Hardy- Weinberg's law is important primarily because it describe the situation in which there is genetic equilibrium and no evolution.

1. It provides a theoretical baseline for measuring evolutionary change.
2. The equilibrium tends to conserve gains which have been made in the past and also to avoid too rapid changes.
3. Equilibrium maintains heterozygosity in the population.
4. Equilibrium prevents evolutionary progress.