CHAPTER

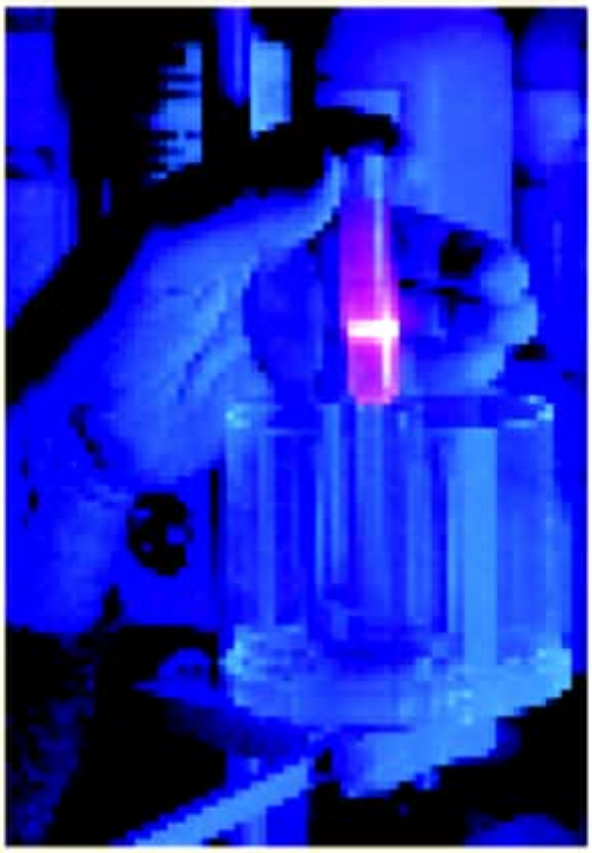
9

Genetic Engineering

(Isolation, Sequencing, Synthesis of Gene and DNA Fingerprinting)

Allaby (1995) has defined the genetic engineering as the modification of the genetic information of living organisms by direct manipulation of their DNA (rather than by the more indirect method of breeding). Thus, a gene of known function (or economic importance) can be transferred from its normal location into a cell (which originally lack it) via a suitable mobile genetic element, called **vector** (such as plasmid, viruses (phages), etc.). The transferred gene replicates normally and is handed over to next progeny. On confirmation for its presence through biochemical procedures, the replica of the same cell (*i.e.*, clones) can be produced. With genetic engineering (also called **gene cloning**, **recombinant DNA technology** or **gene manipulation**), thus, genes can be isolated, cloned and characterized. More recently, **polymerase chain reaction** (**PCR**) which involves a thermostable DNA polymerase enzyme (*e.g.*, **Taq polymerase**) has also been used to obtain millions of copies of DNA segments (or genes) of choice.

The techniques of recombinant DNA and gene cloning are most powerful tools ever developed in the field of biology. The technique of designed genetic engineering of living cells has many potential applications. If used wisely, it promises to



The future of the Earth's great diversity of life may eventually depend on genetic engineering, which has made it possible to extract and store the DNA of whole organisms indefinitely.

enhance the quality of human life. However, if used hapazardly and carelessly, genetic engineering could have negative impact on the quality of our life.

Genetic engineering is the 'hot cake' of todays' high-tech world; it has been applied for the production of valuable polypeptides, insulin, interferon, growth hormones and of course in the transfer of *Nif* (=nitrogen fixing) genes and control of genetic diseases (*e.g.*, cancer). Genetic engineers have promised a free agriculture from constraining requirements for fertilizers and pesticides. In this chapter some of the important techniques of genetic engineering will be described.

TOOLS OF GENETIC ENGINEERING

There are various biological tools which are used to carry out manipulation of genetic materials and cells as well. Some of them have been described as follows :

1. Enzymes such as exonucleases, endonucleases, restriction enzymes (=restriction endonucleases), SI enzymes (to change cohesive ends of single stranded DNA fragments into blunt ends), DNA ligases, alkaline phosphatase, reverse transcriptase, DNA polymerases.

2. Foreign DNA/Passenger DNA. It is a fragment of DNA molecule which is enzymatically isolated and cloned. The gene is identified on a genome and pulled out from it either before or after cloning. The cloned foreign DNA fragment expresses normally as in parent cell.

3. Cloning vectors. Vectors or vehicle DNA are those DNA that can carry a foreign DNA fragment when inserted into it. Based on the nature and sources, the vectors are grouped into bacterial plasmids, bacteriophages, cosmids and phasmids.

Plasmids have various curious properties : 1. The genes they carry may not be absolutely essential for life and so a plasmid can sometimes leave one bacterial cell and enter another, thereby transferring genetic traits between cells. 2. The plasmid can reproduce itself inside the bacterium independently of the main bacterial DNA. 3. A plasmid can sometimes fuse with the main DNA and later on can depart from the main genome, but in such a manner as to drag a piece of the main DNA with it. Nature seems to have evolved plasmid as an efficient way of exchanging gene between bacterial cells (see **Nossal** and **Coppel**, 1989).

4. cDNA bank or cDNA library. DNA copy of an mRNA molecule is known as **copy DNA** or **cDNA** (also called **complementary DNA**). The well characterized cDNA molecule is allowed to bind with a suitable vector which then **transforms** a bacterial cell in such a way that it does not disrupt its normal function. The transformed bacterial cell containing a plasmid with DNA copy of an mRNA molecule is known as **cDNA clone**. Since it is difficult to get cDNA from the double stranded DNA molecules, therefore, most of the cDNA clones are prepared by the use of **reverse transcriptase enzyme** from mRNA sequences of eukaryotic cells (Fig. 9.1).

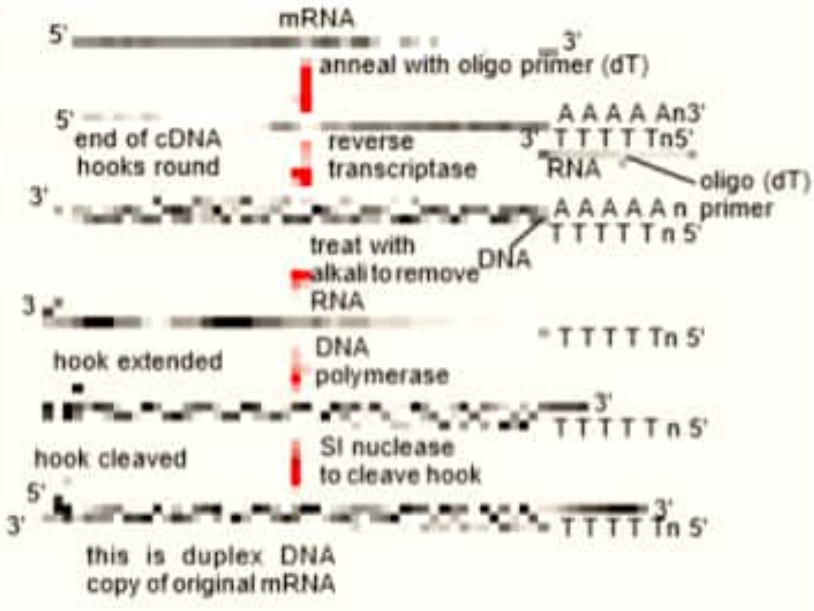


Fig 9.1. Synthesis of cDNA from mRNA, using reverse transcriptase enzyme.

A researcher analyzes the results of a DNA sequencing experiment and enters them directly into a computer. These are submitted to gene bank so that fellow researchers can have ready access to it.

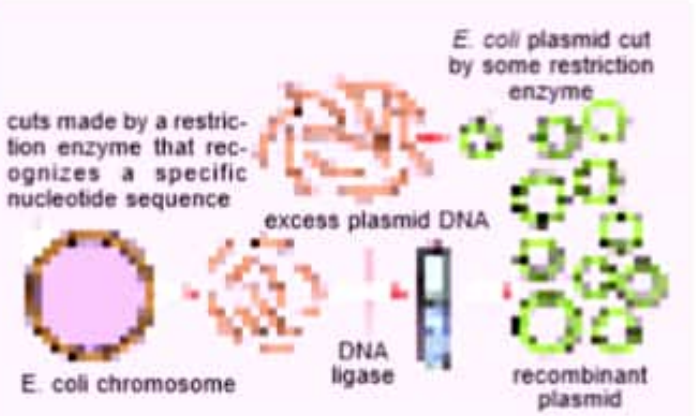


Fig. 9.2. Technique of formation of a genomic library using recombinant DNA technique.

5. Genomic library. Gene bank or **genomic library** is a complete collection of cloned DNA fragments which comprises the entire genome of an organism (**Dahl** *et al.*, 1981). Genomic library is constructed by a **shotgun experiment** where whole genome of a cell is cloned in the form of random and unidentified clones. The cloned DNAs are produced by (1) isolation of DNA fragments to be cloned; (2) joining the fragments to a suitable vector (usually phage λ) ; (3) introduction of recombinant DNA into host cells at high efficiency to get a large number of independent clones; (4) selection of the desired clones; and (5) use of clones for the construction of genome bank (Fig. 9.2 and Fig. 9.3).

6. Molecular probes. A probe is either a radioactive labelled (^{32}P) or non-radioactive labelled (*viz.*, biotin or digoxigenin), single stranded nucleic acid (20–40 nucleotide long) with a sequence complementary to at least one part of the desired DNA. The probe may be partially pure mRNA, a chemically synthesized

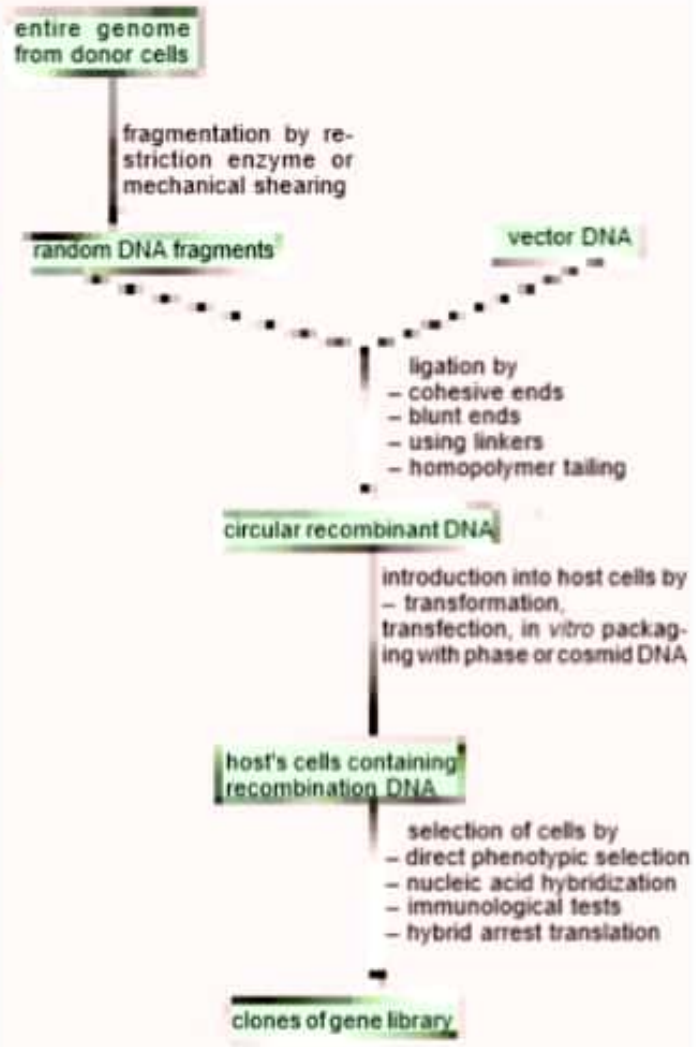


Fig. 9.3. Methods of construction of gene library.

oligonucleotide or a related gene which identifies the corresponding recombinant DNA. DNA/RNA probes have been commercially exploited in the diagnosis of infectious diseases, a variety of microbiological tests, identification of food contaminants and in forensic tests (*e.g.*, fingerprinting of murderers and rapists). Antibodies are also occasionally used as probes to recognize specific protein sequences (see **Dubey**, 1995).

CERTAIN GENERAL TECHNIQUES OF GENETIC ENGINEERING

The opening move of the genetic engineer is to break the DNA up into small, manageable bits, each containing one or just a few genes. Each little bit, one at a time, is stitched into a special, virus-like piece of DNA gifted with the ability for self-replication. These virus-like, recombined DNA molecules now invade rapidly-dividing host cells, again one at a time. Each host cell (*e.g.*, a bacterium or yeast) thereby becomes a factory for one pure gene. Clever tricks allow the genetic engineer to pick out the host cell carrying the gene wanted for that particular experiment. By isolating that one special cell and growing it up to any desired quantity, the one desired gene (or its protein product) can be obtained.

Now let us consider some of the general methods of genetic engineering as follows :

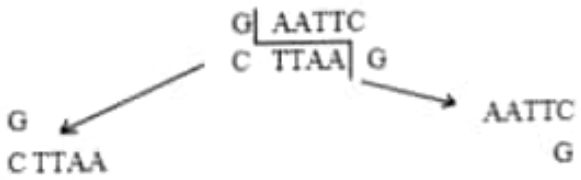
1. Isolation and Use of Restriction Enzymes

Recombinant DNA technology makes a frequent use of restriction endonucleases which cut the DNA double helix in very precise ways. They have the capacity to recognize specific base sequences on DNA and then to cut each strand at a given place. These enzymes are called **restriction enzymes** because they restrict infection of bacteria by certain viruses (*i.e.*, bacteriophages), by degrading the viral DNA without affecting the bacterial DNA. Thus, their function in the bacterial cell is to destroy foreign DNA that might enter the cell. The restriction enzyme recognizes the foreign DNA and cuts it at several sites along the molecule. Each bacterium has its own unique restriction enzymes and each enzyme recognizes only one type of sequence. As already described elsewhere, the DNA sequences recognized by restriction enzymes are called **palindromes**. Palindromes are the base sequences that read the same on the two strands but in opposite directions. For example, if the sequence on one strand is GAATTC read in 5'→3' direction, the sequence on the opposite strand is CTTAAG read in the 3'→5' direction, but when both strands are read in the 5'→ 3' direction the sequence is the same. The palindrome appears accordingly —

In recombinant protein research, a scientist takes a sample in which genetically engineered mammalian cells are being cultured.

5' GAATTC 3'
3' CTTAAG 5'

In addition, there is a point of symmetry within the palindrome. In our example, this point is in the centre between the AT/AT. The value of restriction enzymes is that they make cuts in the DNA molecule around this point of symmetry. Some enzymes cut straight across the molecule at the symmetrical axis producing **blunt ends**. Of more value, however, are the restriction enzymes that cut between the same two bases away form the point of symmetry on two strands, thus, producing a staggered break.



In this example, we have used the palindrome sequence recognized by one of the most popular restriction enzymes, called **Eco RI** from *E.coli* (bacterium). Hundreds of other restriction enzymes with different sequence specificities have been isolated from several bacteria and are commercially available.

The most useful aspect of restriction enzymes is that each enzyme recognizes the same unique base sequence regardless of the source of the DNA. It means that these enzymes establish fixed landmark along an otherwise very regular DNA molecule. This allows dividing a long DNA molecule into fragments that can be separated from each other by size with the technique of gel electrophoresis (*e.g.*, **agarose** or **polyacrylamide gel electrophoresis**, **pulsed field gel electrophoresis** or **PFGE**). Each fragment is, thus, also available for further analysis, including the sequencing.

One value of cutting DNA molecule up into discrete fragments is being able to locate a particular gene on the fragment where it resides. This is done by the general technique of **Southern blotting** (developed by **E.M. Southern**, 1975).

1. Southern blotting technique. In this technique, a DNA molecule is cut into discrete fragments by a restriction enzyme. It is electrophoresed through an agarose gel which separates the various fragments according to size. The DNA is then denatured into single strands by exposing the gel to NaOH. A few pieces of filter paper soaked in buffer are placed under the gel. A large piece of nitrocellulose paper is laid over the agarose gel, followed by several layers of absorbent material such as filter paper. This dry absorbent material pulls the buffer up through the gel from the lower layer (Fig. 9.4). This washes the DNA off the gel and on to the filter, where it covalently binds to the filter.

The positions of the DNA molecules on the filter paper are identical to their position in the gel. The nitrocellulose filter containing the DNA is first dried and then exposed to a solution of ^{32}P labelled mRNA called **molecular probe** from the gene to be isolated. The radioactive mRNA hybridizes (*i.e.*, establishes the hydrogen bonds) only with the single-stranded DNA in restriction fragments that contain complementary sequences. The nitrocellulose filter is then removed and placed in contact with photographic film that when developed will reveal fragments from the original gel containing complementary sequences to the mRNA used in the assay. The procedure allows specific identification of restriction fragments containing DNA sequences to specific RNA molecules.

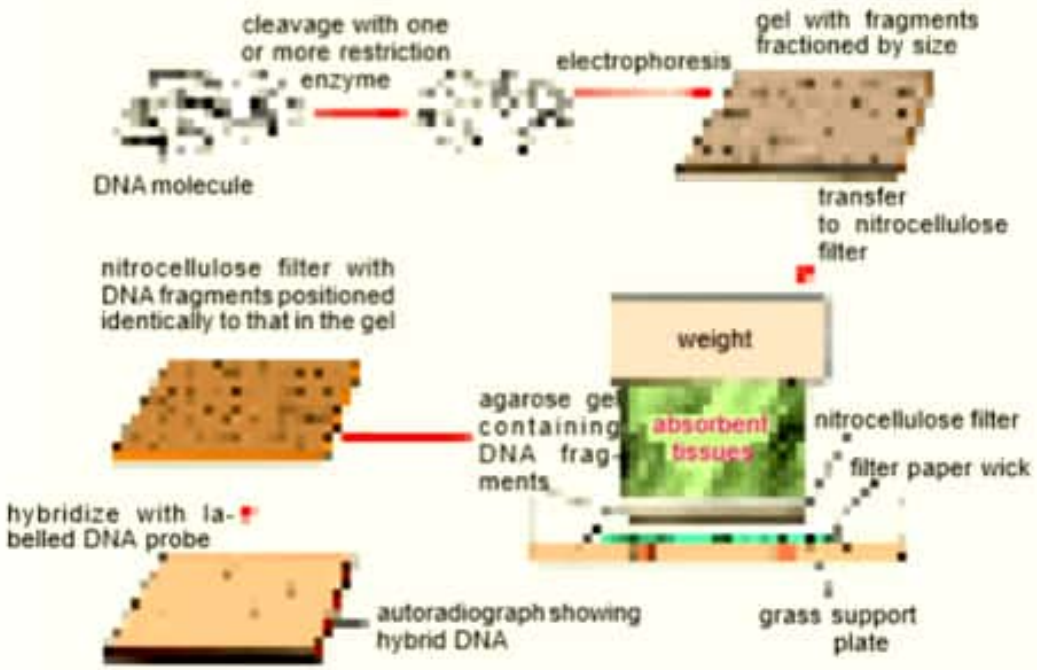
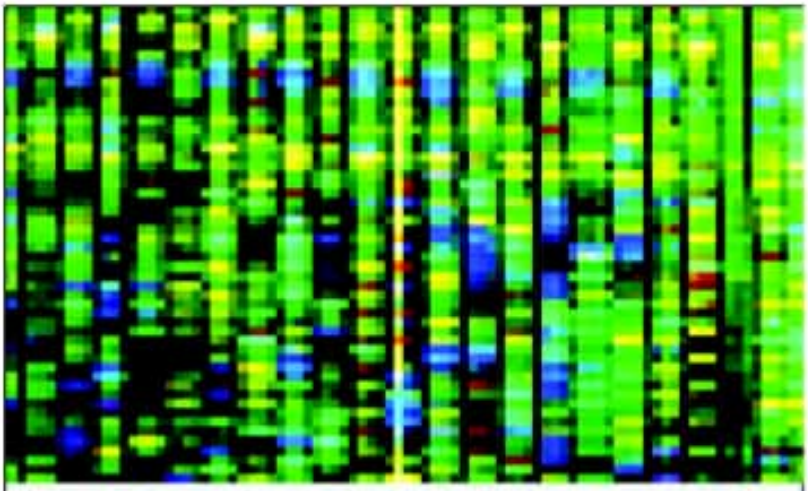


Fig. 9.4. Various steps of Southern blotting technique.



An autoradiogram is used to decode the base sequence after electrophoresis.

2. Northern blotting technique. Alwine *et al.*, (1979) devised a technique, nicknamed **northern blotting** in which RNA bands are blot transferred from the gel onto chemically reactive paper. An **amino- benzyloxymethyl paper** which is prepared from Whatman filter paper No. 540 after a series of simple reactions, is **diazotized** (Gr., *di*= two; Fr., *azote* = nitrogen; to introduce the diazo group into a chemical compound, usually through the treatment of an amine with nitrous acid) and made into the reactive paper and, therefore, becomes available for hybridization with radiolabelled DNA probes. The hybridized bands are found out by autoradiography.

Later, it was shown (**Tomas**, 1980) that mRNA bands can be blotted directly onto nitrocellulose membrane, a technique which has been widely adopted. The mRNA bands blotted onto nitrocellulose membrane can be hybridized with a labelled DNA or RNA probe. The single stranded regions of probe are removed by nuclease (*e.g.* mung bean nuclease or S-1 nuclease), so that quantitative estimation of hybridized mRNA can also be made.

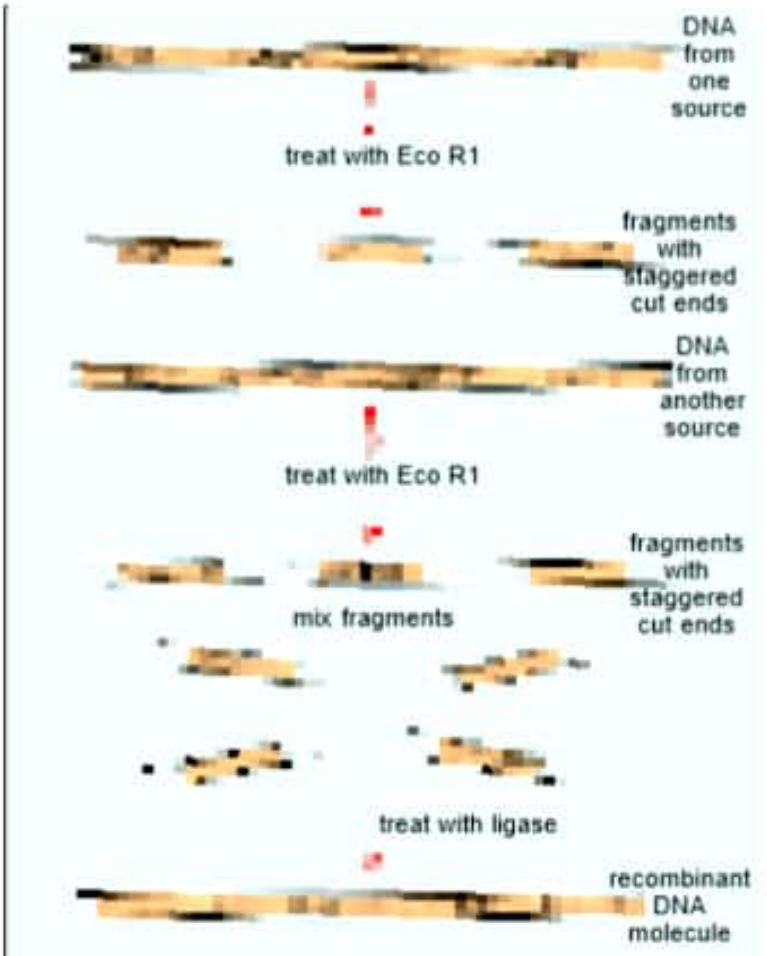


Fig. 9.5. Method of construction of a recombinant DNA molecule using a restriction enzyme.

3. Western blotting technique. Towbin *et al.*, (1979) developed the **western blotting technique** to find out the newly encoded protein by a transformed cell. The extracted proteins are subjected to **polyacrylamide gel electrophoresis** (**PGE**) and are then transferred onto nitrocellulose to which they bind. Nitrocellulose membrane is then used for probing with a specific labelled antibody (Antibody tends to bind with a protein; it does not hybridize with protein). The antibody may be labelled with ^{125}I and the signal is detected again with autoradiography.

2. Vectors, Transformation and Molecular Cloning

Plasmids are extra-chromosomal DNA elements found mostly in bacteria. These plasmids contain DNA sequences coding for drug resistance, sex factor (F factor) etc., and probably has arisen from chromosomal DNA. When the bacterium multiplies, the plasmid DNA will also multiply along

with chromosomal DNA. It is possible to isolate these plasmids in large quantity. In recombinant DNA technology, prokaryotic and eukaryotic DNAs as well as the plasmid DNAs are cut into specific fragments with restriction enzymes. The foreign DNA fragment (prokaryotic and eukaryotic) can be made to recombine with the plasmid DNA and the product is referred to as **recombinant DNA**. The plasmid DNA carrying the foreign DNA fragment can be put back into a suitable recipient bacterium. This bacterium can be grown in large quantities and the recombinant plasmids are isolated from such bacteria. (This is called **molecular cloning**). The foreign genes then can be released from recombinant plasmids once again by the use of restriction enzymes. Thus, large number of foreign genes can be isolated by this technology.

The foreign genes can also be introduced into viral DNAs and when such recombinant viruses (phages) infect and multiply, once again large quantities of desired genes can be isolated. Plasmids and viruses which are used as carriers of foreign DNAs are referred to as **vectors** or **vehicle DNAs**. By interchanging plasmid DNA and viral DNA fragments, several new vectors have been synthesized which carry new genes into bacteria, yeast, insect, plant and animal cells. When such new (foreign) DNA fragments are introduced into relevant host cells, such cells are said to be **transformed** (in case of bacteria) or **trangenic** (in case of plants and animals) and the process is called **transformation**. In animals the term **transfection** is used in place of transformation. For example, **D.W.OW** *et al.*, (1986) produced a transgenic tobacco plant harbouring the luciferase gene of the firefly (see **Gardner** *et al.*, 1991).

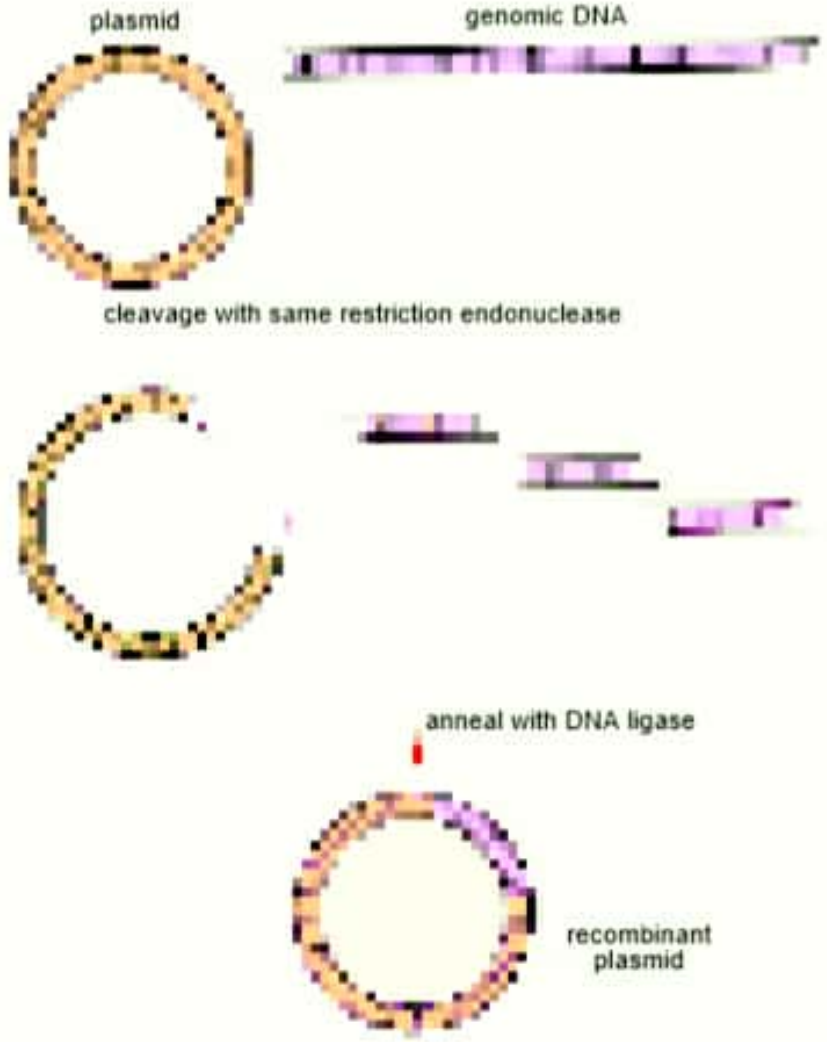


Fig. 9.6. Method of construction of a recombinant plasmid between a bacterial plasmid and genomic DNA from another organism.

ISOLATION OF GENES

The first gene to be isolated was the **lac operon** of *E.coli* by **Shapiro** and his colleagues in 1969. However, in recent years great progress has been made in the techniques for isolation of a variety of genes, some examples of which are the following: (i) ribosomal RNA ; (ii) specific protein products; (iii) phenotypic traits with unknown products; and (iv) genes for regulatory functions, *e.g.*, promoter gene, etc. For each of these genes different technique was used. We can consider here in detail the following technique :

Isolation of Ribosomal RNA Genes in *Xenopus*

As already described elsewhere, ribosomes consist of ribosomal proteins and ri-

bosomal RNA (rRNA). Ribosomal RNA makes 80 per cent of cellular RNA and occurs in four sizes namely 28S, 18S, 5.8S and 5S. The rRNA is synthesized on ribosomal genes which have been isolated. Isolation of rRNA genes have been found easy due to the following three reasons: 1. availability of homogeneous rRNA; 2. differences between ribosomal RNA genes and other genes, *i.e.*, rRNA has high G+C content, *i.e.*, rRNA has 45 to 60 per cent G+C; while the remaining RNA has only 40 per cent G+C; 3. rRNA genes are present in multiple copies. Due to these facts, rRNA genes were isolated in 1965 in *Xenopus* by **Hugh Wallace** and **Max L. Birnstiel**. The technique of isolation of rRNA genes involved the following steps: (1) The rRNA was isolated from ribosomes of *Xenopus* and made radioactively labelled due to its replication in a medium having tritiated uridine. (2) Ribosomal DNA was isolated by density gradient centrifugation followed by its denaturation (since G+C content of rDNA differs from that of bulk DNA, it helps in its separation by centrifugation). (3) Single-stranded DNA was fixed on a filter paper. (4) Labelled rRNA was added on filter paper carrying single stranded DNA. (5) DNA-RNA hybridization was allowed to take place. (6) Excess labelled RNA was washed. (7) Radioactivity was measured and duplex hybrids isolated, which on denaturation, gave single stranded DNA, which could be made double stranded.

SEQUENCING OF GENE

Once a gene or DNA fragment is cloned, its further study involves DNA sequencing. The following three methods are used for the determination of DNA sequences :

1. Maxam and Gilbert's Chemical Degradation Method

As illustrated in Figure 9.7, this technique involves the following steps : 1. The 3' ends of DNA are labelled with ^{32}P. 2. The two strands of this radioactively labelled DNA are separated. 3. The mixture is divided into four samples, each treated with a different reagent having the property of destroying either only G, or only C, or A and G or T and C. The concentration of the reagent is adjusted in such a way that 50 per cent of target base is destroyed, so that fragments of different sizes having ^{32}P are produced. 4. Each of the four samples is electrophoresed in four different lanes of the gel. 5. The gel is autoradiographed to determine the sequence from positions of bands in four lanes.

2. Sanger's Dideoxynucleotide Synthetic Method

Fred Sanger had initially developed a method for DNA sequencing, which utilized DNA polymerase to extend DNA chain length. This was termed as **plus-minus method**. Later on, **Sanger** (1986) developed a more powerful method, utilizing single-stranded DNA as the template for DNA synthesis, in which **2', 3' dideoxynucleotides** were incorporated leading to termination of DNA synthesis. These dideoxynucleotides are used as triphosphates (ddNTP) and can be incorporated in a growing chain, but they terminate synthesis (Fig. 9.8), since they fail to form a phosphodiester bond with next incoming deoxynucleotide triphosphate (dNTP). Thus, Sanger's dideoxy methods includes the following steps : 1. Four reaction tubes are set up, each containing single stranded DNA sample (cloned in M13 phage) to be sequenced, all four dNTPs (radioactively labelled) and an enzyme for DNA synthesis (*i.e.*, DNA polymerase I). Each tube also contains a small amount of one of the four ddNTPs, so that each tube has a different ddNTP, bringing about termination at a specific base — adenine (A), cytosine (C), guanine (G) and thymine (T). 2. The DNA fragments which are generated by random incorporation of ddNTP leading to termination of reaction are then separated by electrophoresis on a high resolution polyacrylamide gel. This is done for all the four reaction mixtures on adjoining lanes in the gel. 3. The gel is used for autoradiography so that the position of different bands in each lane can be visualized. 4. The bands on the autoradiogram can be used for getting the DNA sequence.

3. Direct DNA Sequencing Using PCR

Polymerase chain reaction (PCR) has also been used for sequencing the amplified DNA product (Fig. 9.9). This method of DNA sequencing is faster and more reliable. It can utilize either the whole genomic DNA or the cloned fragments for sequencing a particular DNA segment. The DNA sequencing using PCR involves two main steps : 1. generation of sequencing templates (double stranded or single stranded) using PCR and; 2. sequencing of PCR products either with the thermostable Taq DNA polymerase. PCR is discovered by **Kary Mullis**, 1985, and nicknamed **people's choice reaction** in which instead of RNA primer, a deoxyolig onucleotide is used. (In PCR reaction, the normal DNA polymerase enzyme is replaced by **Taq DNA polymerase** (= an enzyme isolated from *Thermus aquaticus* growing in hot springs; this enzyme acts best at 72°C and the denaturation temperature of 90°C does not destroy its enzymatic activity, see **Kary Mullis**, 1990). An unlimited supply of amplified DNA is obtained by repeating the reaction, which is made possible by regular denaturation of freshly synthesized double stranded DNA molecules by heating it to 90° – 98°C. At this high temperature the two DNA strands separate. Once the double stranded DNA is made single stranded by heating up to 90 – 98°C, the mixture with two primers (= deoxyoligonucleotides) recognizing the two strands and bordering the sequence to be amplified, is cooled to 40 – 60°C. This permits the primers to bind to their complementary strands through renaturation. The presence of Taq DNA polymerase enzyme and all four essential nucleoside triphosphate in the 'eppendorf tube' allows synthe-

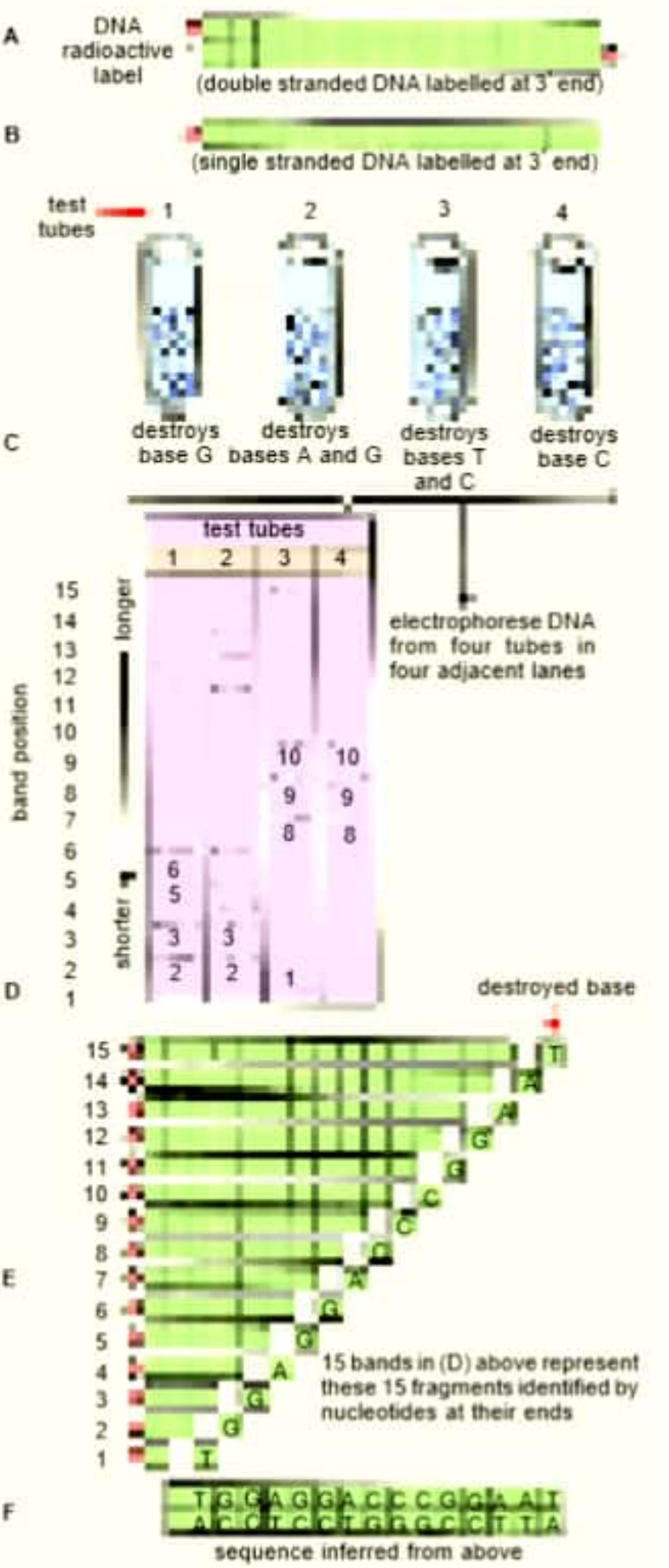


Fig. 9.7. Maxam and Gilbert's chemical degradation method for sequencing of DNA.

sis of complementary strands in the usual manner. In an automatic thermal cycler, this process is automatically repeated 20 – 30 times (as predetermined by a computer device), so that in a single afternoon a billion copies of the sequence flanked by the left and right primers, can be produced (Fig. 9.9). In order to continue the synthesis, the temperature of the mixture is alternately increased (for denaturation) and decreased (for renaturation) once every 1-3 minutes as fixed by the computer device). The use of Taq DNA polymerase and some other DNA polymerases have allowed automation of the entire PCR reaction.

In fact, the DNA sequencing method using PCR eliminates the need of cloning the DNA in single stranded DNA phage vector, *i.e.*, M13.

SYNTHESIS OF GENE

The genes can be synthesized by the following two methods :

1. Organochemical Synthesis of Polynucleotides (or Chemical Synthesis of tRNA Genes)

When the detailed structure of a gene becomes procurable, then such a gene can be synthesized by a purely chemical method. The structure of gene could be inferred from its product. For instance, if a gene is responsible for giving rise to a polypeptide chain and the structure of the chain is known, then from the genetic code dictionary, structure of the gene could be easily inferred. Such genes were initially considered to be too long to be synthesized, because an average gene contains about 1500 base pairs. But since tRNA molecules are fairly small in size (about 80 nucleotides), a gene responsible for giving rise to a tRNA molecule was found to be within the reach of synthesis.

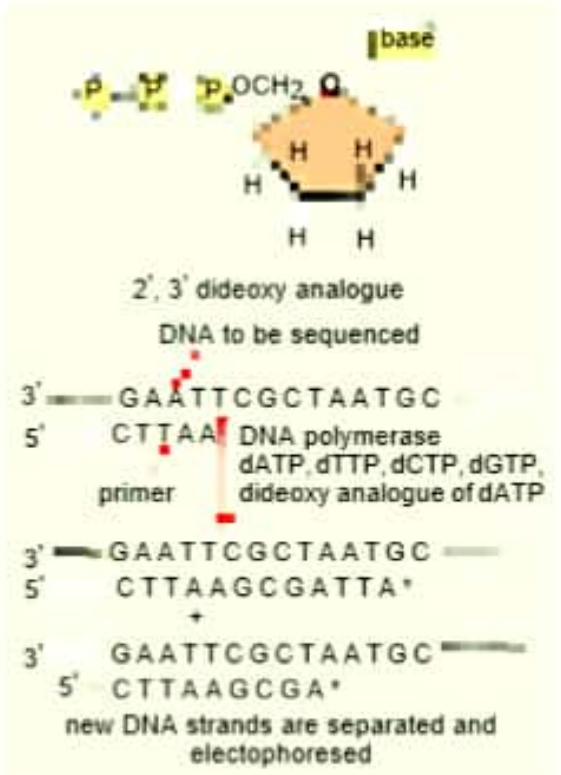


Fig. 9.8. A– Dideoxynucleotide; B– Technique involved in Sanger's chain termination method for sequencing of DNA.

Gene machines are just automated chemistry sets. The machine pumps a precise amount of one of four solutions of the bases of DNA contained in the jar along fine pipes to a reaction chamber. A computer controls which base is added. The base is added chemically to a growing chain of DNA. The addition cycle repeats until the entire sequence is made.

(i) Synthesis of gene for yeast alanyl tRNA. R.W. Holley (who died in 1993) and his coworkers (1965), first of all gave the detailed structure of yeast **alanyl tRNA** (containing 77 nucleotides; see Chapter 5). **Khorana** and his coworkers had vast experience of synthesizing DNA of known base sequences. They found that such a long chain (*i.e.*, 77 base pairs of DNA of yeast alanyl tRNA) could not be synthesized by adding a single base each time, therefore, they decided that small oligo-deoxyribonucleotides ranging in length from 5 to 20 nucleotides should first be synthesized. These segments would be single stranded and would cover the whole length of both the strands of DNA.

These would then be joined to form double stranded DNA, 77 nucleotide pairs long. Thus, the process of synthesis of gene for yeast alanyl tRNA involves the following steps :

(a) Synthesis of oligonucleotides. Fifteen oligonucleotides ranging from pentanucleotide (5 bases) to an icosanucleotide (20 bases) were synthesized (Table 9-1). Such sort of synthesis was conducted through condensation between hydroxyl group at the 3' position of one nucleotide and phosphate group at 5' position of the second nucleotide. In order to bring about condensation, all other functional groups, not taking part in condensation, were protected using specific protective groups. After protecting the groups, reaction between a nucleotide with protected 5' end and another nucleotide with protected 3' end proceeded according to Figure 9.10. Subsequent condensation was done between groups of two, three or four nucleotides.

(ii) Synthesis of three duplex fragments. Fifteen single-stranded oligonucleotides were used to prepare three duplex fragments, each containing a single stranded end (Fig. 9.14). These three fragments are characterized as follows :

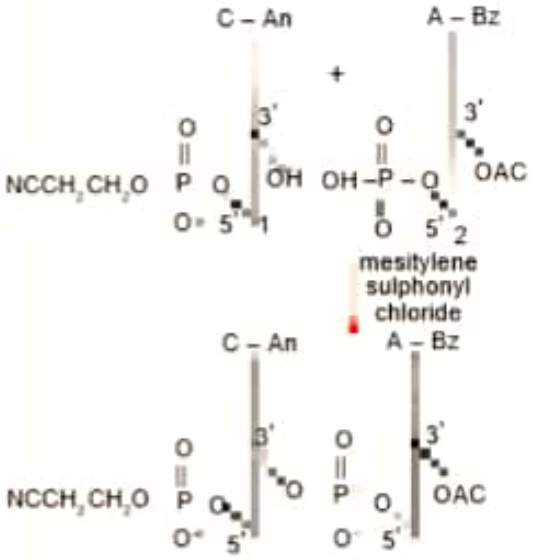


Fig. 9.10. Mode of condensation between two nucleotides with protected 5' OH and 3' OH groups in the sugars and protected amino groups in the nitrogen bases (AC=acetyl, An=anisoyl and B_z= benzoyl protective groups).

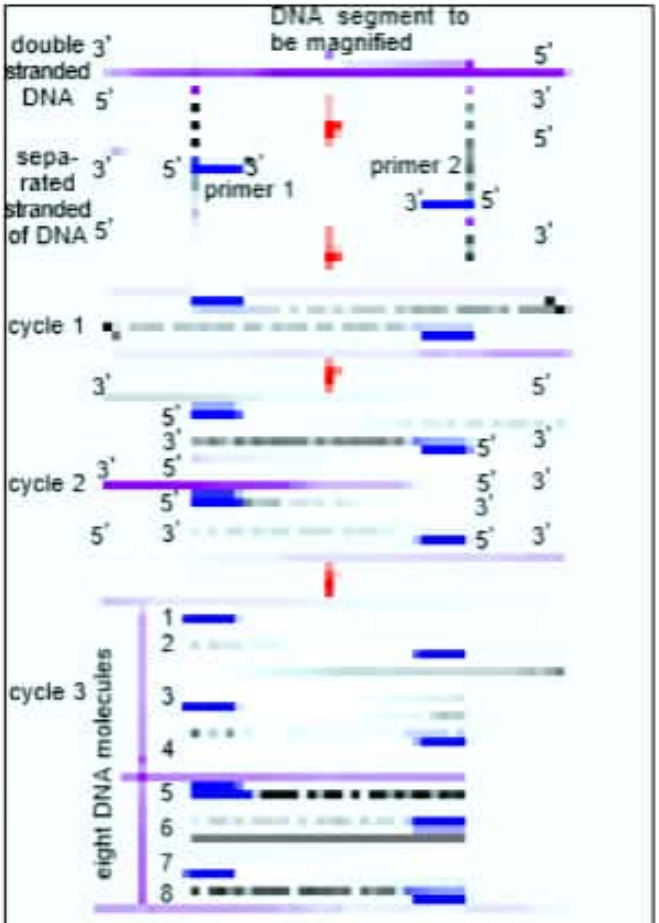


Fig. 9.9. Steps involved in basic polymerase chain reaction (PCR); only three cycles of PCR are shown; in each cycle primers are shown by solid boxes, template strands are shown by continuous lines and newly synthesized strands are shown by broken lines.

1. **Fragment A** consisted of the first 20 nucleotides in which nucleotides 17–20 being single stranded. 2. **Fragment B** contained nucleotide residues from 17 to 50, in which single stranded region being 17–20and 46–50. 3. **Fragment C** included nucleotide residues 46–77 with single stranded region 46–50.

(iii) Synthesis of tRNA gene from three duplex fragments. In the concluding step three duplex fragments A, B and C were joined (linked by ligase enzyme) to give complete gene in each of the following two ways :

1. In one scheme, A fragment was joined to B fragment taking advantage of the overlap in residues 17–20; fragment C was then added, with the overlap in the region of 46–50 residues. The complete double stranded DNA with 77 base pairs representing the gene was, thus, prepared. 2. In the alternative scheme

B fragment was first added to C fragment and to this A fragment was added in the end to get the complete gene. This gene was synthesized in 1970. Since such a synthetic gene could replicate and make its own copies, so, was used for subsequent work.

In 1977, **Riggs** and his colleagues have been able to synthesize a DNA piece (*i.e.*, a gene) which codes for the polypeptide (containing 14 amino acids) of **somatostatin**. This hormone regulates body growth as well as the production of insulin and glucagon hormones and also inhibits the release of other pituitary hormones in mammals.

Synthesis of complete gene. During the synthesis of the gene or yeast alanyl tRNA, it became clear that natural tRNA was not the direct product of transcription. Instead a precursor molecule is synthesized which subsequently, after losing segments of RNA by cleavage, gives rise to tRNA. This has shown that the real natural gene for yeast alanyl tRNA was longer than the DNA duplex synthesized by Khorana. In view of this **Khorana** started the synthesis of a gene for *E.coli* **tyrosine suppressor tRNA precursor**. In 1979, he reported the completion of total synthesis of a biologically functional tyrosine suppressor transfer RNA gene carrying all regulatory sequences (Fig. 9.12). This gene was 207 base pairs long and included the following components : (i) a 51 base pairs long DNA promoter region; (ii) a 126 base pairs long DNA corresponding to the precursor tRNA and (iii) 25 base pairs long DNA corresponding to 16 base pairs adjoining CCA end of tRNA and the remainder, a modified sequence including

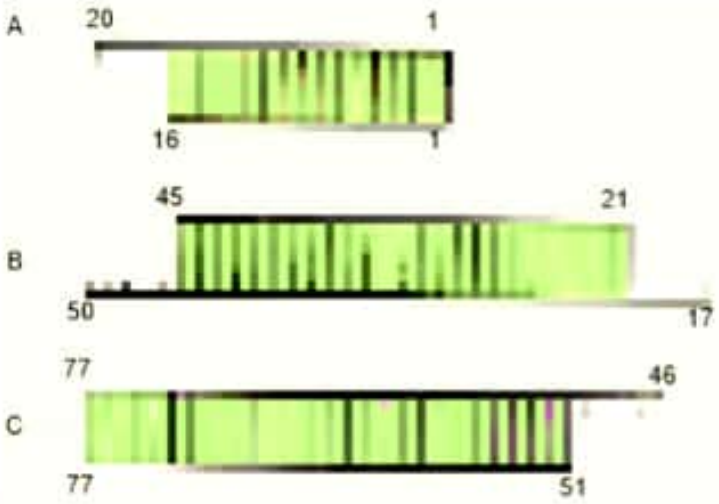


Fig. 9.11. Three duplex DNA fragments with single stranded sticky ends, produced for the synthesis of yeast alanyl tRNA gene by H.G. Khorana.

Table 9.1. **Base sequences and lengths of the single-stranded oligonucleotides synthesized for the construction of yeast alanine tRNA gene.**

Serial number	Base sequence	Length of nucleotides
1.	1 2 3 4 5 6 7 8 9 10 11 12 T G G T G G A C G A G T	12
2.	1 2 3 4 5 6 A C C A C C	6
3.	1 2 3 4 5 6 7 8 9 10 T G C T C A G G C C	10
4.	1 2 3 4 5 6 7 8 C C G G A A T C	8
5.	1 2 3 4 5 6 7 8 9 10 11 T T A G C T T G G C C	11
6.	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 T A A C C G G A G A G A C T C C C A T G	20
7.	1 2 3 4 5 6 7 T C T C T G A	7
8.	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 G G G T A C G A A A C C C T C G	16
9.	1 2 3 4 5	5

	C T T T G	
10.	1 2 3 4 5 6 7 8 9 10	10
	G G A G C G C G C T	
11.	1 2 3 4 5 6 7 8 9 10	10
	C G C G A T G G C T	
12.	1 2 3 4 5 6 7 8 9 10	10
	A C C G A C T A C G	
13.	1 2 3 4 5 6 7 8 9 10	10
	G A T G C G C G G T	
14.	1 2 3 4 5 6 7 8 9 10 11 12	12
	C G C C A C A C G C C C	
15.	1 2 3 4 5 6 7	7
	G T G C G G G	

Eco RI endonuclease specific sequence. This complete synthetic gene was cloned in the vector bacteriophage (lambda phage) by gene cloning technique. On transformation of *E. coli*, the phage could multiply with the cloned gene.

2. Synthesis of Gene from mRNA (or Enzymatic Synthesis of Gene)

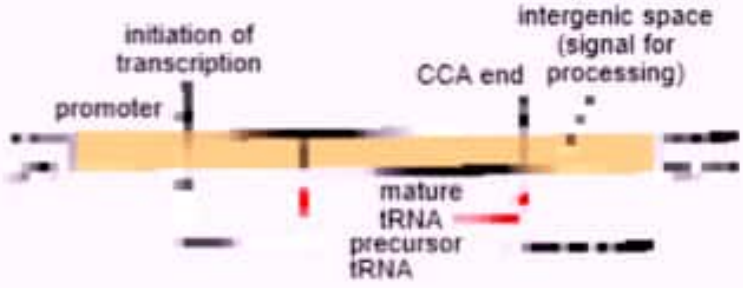


Fig. 9.12. Different parts of the complete *E. coli* tyrosine suppressor tRNA gene, synthesized by Khorana.

RNA directed DNA polymerase enzyme, which was discovered by **Temin** and **Baltimore** (1970), can synthesize DNA from RNA template. This enzyme has enabled the molecular biologists to synthesize complementary (or copy) DNA (cDNA) using mRNA as a template. If mRNA transcribed from a specific gene is made available in purified form, it can be used in the production of cDNA which will represent the synthetic gene. Thus, by copying eukaryotic purified mRNA, several genes have been artificially synthesized. Most important of these genes are the genes for sea urchin histone proteins, ovalbumin gene in chicken and globin genes in mammals.

One of the leading discoveries, in which the technique of enzymatic synthesis of gene has been utilized, is the synthesis of rat **insulin cDNA** segments on insulin mRNA isolated from pancreatic islets of Langerhans (**Ullrich** *et al.*, 1977). This cDNA has been replicated to get multiple copies of the rat insulin gene. Further, after isolation and purification of mRNA from the cultured rat pituitary cells, **Seeberg** *et al.* (1977) has synthesized cDNA on mRNA that codes for a **rat growth hormone** (**RGH**) which comprises 190 amino acids. Similarly, a cDNA molecule has been synthesized on mRNA which codes for the **human chorionic somatomammotropin** (HCS), a placental lactogen (**Shine** *et al.*, 1977).

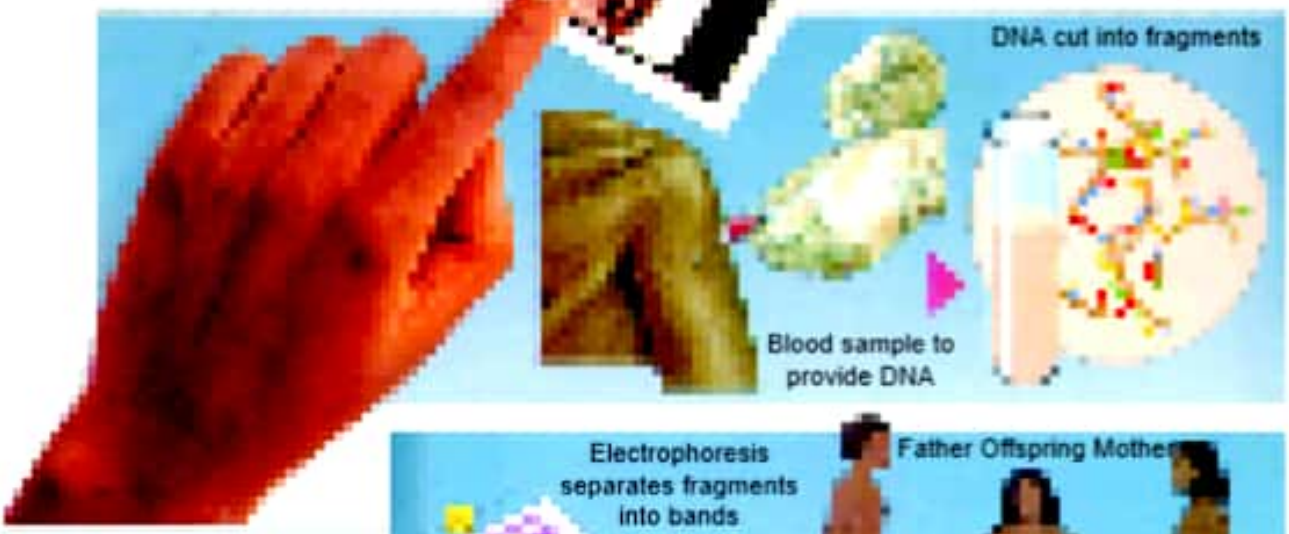
APPLICATION OF GENETIC ENGINEERING

The foremost application of genetic engineering has been in understanding the structure of eukaryotic genes such as exons, introns, promoters, enhancers, silencers, etc. Some other modern applications of genetic engineering include — (i) engineering of bacteria to carry out specific processes or to produce important molecules such as hormones or antibiotics; (ii) altering the genotypes of plants as an aid in plant breeding, *e.g.*, formation of insect resistant tomato plants; (iii) altering genotypes of animals to correct their genetic defects, *e.g.*, production of human growth hormones.

DNA Fingerprinting : The Ultimate Identification Test

Every year in court cases all over the world the ability to establish a person's identity is essential for a just decision. Genetics has come to the rescue of the courts and now the following new questions are routinely asked in the courts : (1) Is the drop of blood found at the crime scene from suspect on trial? Who is the child's father ? Until recently, there was no foolproof test. In a criminal case, if there was no identifiable fingerprint left behind at the crime scene, there was no case. Blood tests can determine who is not the parent, not who is. A test has now been developed that provides hundred per cent positive identification. The test is called **DNA fingerprinting**. The test of DNA fingerprinting can show conclusively whether the genetic material in a drop of blood matches that of the suspect, or it can be used to solve paternity case.

The technique of DNA fingerprinting relies on developments from recombinant DNA technology and allows an examination of each individual's unique genetic blueprint–DNA. The technique was discovered in England by **Alec Jeffreys**. It is based on the fact that the DNA of each individual is interrupted by a series of identical DNA sequences called **repetitive DNA** or **tandem repeats**. The pattern, length, and number of these repeats are



Some sequences of human DNA vary so greatly that the chances of two people (except identical twins) having the same pattern is one in several million. Genetic or molecular "fingerprinting" provides almost infallible proof of identity and is used as evidence in cases of missing persons, rape, murder and paternity suits.

unique for each individual. **Jeffreys** developed a series of DNA probes, which are short pieces of DNA that seek out any specific sequence they match, and base pair with that sequence. Such molecular probes are used to detect the unique repetitive DNA patterns characteristic of each individual. The procedure of DNA fingerprinting has the following steps : 1. DNA is purified from a small sample of blood, semen, or other DNA-bearing cells, and digested into smaller fragments with restriction endonucleases. 2. The fragments are separated by agarose gel electrophoresis. 3. The separated fragments are transferred to a nylon membrane by the technique of Southern blotting. 4. The DNA probes labelled with radioactive material are added to a solution containing the nylon membrane. 5. Wherever the probes fit a band containing repetitive DNA sequences, they attach. 6. The X-ray film is pressed against

the nylon filter and exposed at bands carrying the radioactive probes attached to the fragments. 7. The patterns of bands obtained on the film is 100 per cent unique for each person, except for identical twins who would have the same pattern.

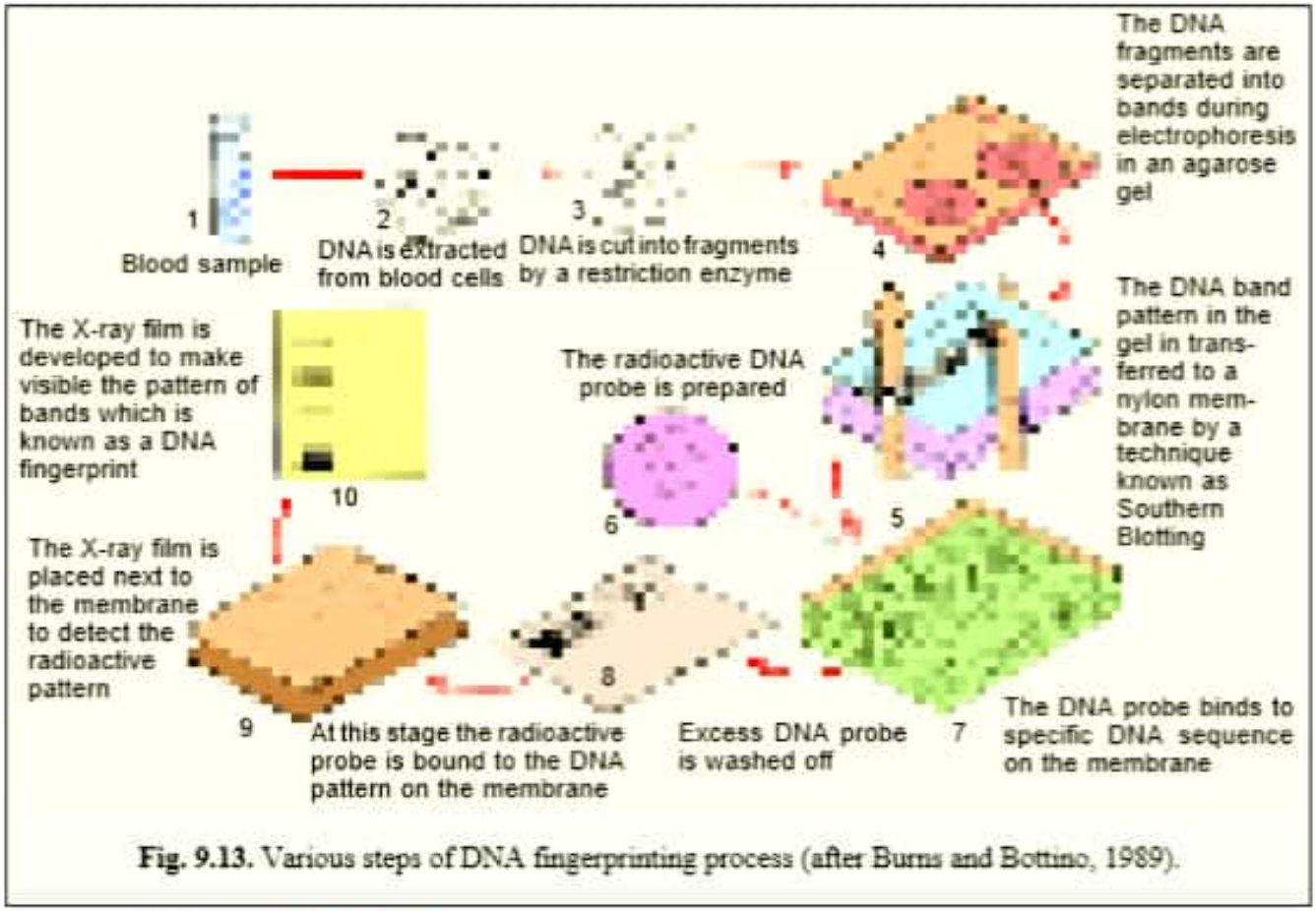


Fig. 9.13. Various steps of DNA fingerprinting process (after Burns and Bottino, 1989).

The **forensic application** of the DNA fingerprinting technique involves a comparison between the DNA fingerprint obtained from cells at a crime scene with a DNA fingerprint from cells provided by the suspect. If the DNA pattern matches exactly, certain identification is made. For **paternity determination**, DNA fingerprints of the mother, child and alleged father are compared. In this case, one-half of the bands in the child comes from the mother and the other half from the father. All the paternal bands in child's DNA fingerprint must match with the alleged father for positive paternity identification.

In India, DNA fingerprinting tests are carried out at the Centre for Cell and Molecular Biology (CCMB), Hyderabad. For this purpose, a test with the **BKM-DNA probe** (= banded krait minor satellite DNA) earlier used for identification of sex chromosomes (by **Dr. Lalji Singh**) has been found to cost one-tenth of the cost of tests used in Europe and U.S.A. Paternity dispute cases are much more common in India and most of them are referred to CCMB for DNA evidence. The first such test on DNA fingerprinting was used in June, 1989 to settle a drawn-out paternity case in Madras.

REVISION QUESTIONS AND PROBLEMS

1. What is genetic engineering ? Describe in brief various essential techniques of genetic engineering.
2. What is recombinant DNA and how is it made ?
3. Why did H.G. Khorana select the gene for yeast alanyl-tRNA for artificial synthesis ? Give a brief account of different steps involved in the artificial synthesis of the gene for yeast alanyl tRNA.
4. Give a brief account of the methods for the isolation of genes in eukaryotes.
5. Enumerate various methods of synthesis of a gene. How can a gene be synthesized from an mRNA molecule ?