KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University Established Under Section 3 of UGC Act 1956)
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DEPARTMENT OF BIOCHEMISTRY

LESSON PLAN

STAFF NAME: Dr. A. MANIMARAN

SUBJECT NAME: CORE ELECTIVE II - INTRODUCTION TO BIOTECHNOLOGY

SUB.CODE: 15BCU603A

SEMESTER: VI **CLASS: III B.Sc., BIOCHEMISTRY**

S.No	Duration of Period	Topics to be Covered	Support material/ Page Nos
		UNIT I	
1	1	Introduction to biotechnology	R1: 3-4
2	1	Overview of Gene cloning	R1: 89-90
3	1	Basic steps in gene cloning	R1: 89-90
4	1	Introduction to Vehicles in gene cloning	R1: 82-86
5	1	Plasmids - basic features	R1: 83-86
6	1	Plasmids - size and copy number	R1: 83-86
7	1	Plasmids - classification	R1: 83-86
8	1	Bacteriophage - basic features	T1: 94-102
9	1	Life cycle of Bacteriophage	R1: 85-86
10	1	Cosmids	T1: 102-103
11	1	Phagemids	T1: 103-104
12	1	Viral vectors	T1: 109-111
13	1	Plant vectors	T1: 109-111
14	1	Revision and Possible QP discussion	
15	1	Revision and Possible QP discussion	
Total No. of Hours planned for Unit I is 15 hours			
		UNIT II	
1	1	Introduction of DNA into living cells	R1: 86-89
2	1	Methods to introduce DNA into living cells	R1: 86-89
3	1	Microinjection	T1: 166-167
4	1	Electroporation	R1: 87-88
5	1	Shotgun methods	R1: 87-88

6	1	Transformation in E.coli	R1: 87-88
7	1	Transfection in E.coli	T1: 121-122
8	1	Recombinant selection and screening	T1: 122-126
9	1	Maximizing the expression of cloned genes in E.coli	W1
10	1	Promoters	T1: 45-46
11	1	Cassettes	
12	1	Fusion protein	R1: 138-139
13	1	Production of fusion protein	R1: 138-139
14	1	Revision and Possible QP discussion	
15	1	Revision and Possible QP discussion	
Total No. of Hours planned for Unit II is 15 hours			
UNIT III			
1	1	Introduction to DNA sequencing	T1: 141-142
2	1	Sanger and Maxim Gilbert method	T1: 141-145
3	1	PCR- techniques and its types	R1: 112-118
4	1	Applications of PCR	R1: 118-119
5	1	DNA foot and finger printing	R1: 185-187
6	1	Applications of DNA foot and finger printing	R1: 187-188
7	1	An overview of Hybridization probes	R1: 125-128
8	1	Radiolabelled Hybridization probes	R1: 125-128
9	1	Non-radiolabelled Hybridization probes	R1: 125-128
10	1	An overview of Hybridization techniques	R1: 97-100
11	1	Southern, Northern, Western blotting techniques	R1: 97-100
12	1	Site directed mutagenesis	R1: 129-133
13	1	DNA microarray	T1: 194-196
14	1	Revision and Possible QP discussion	
15	1	Revision and Possible QP discussion	
Total No. of Hours planned for Unit III is 15 hours			
UNIT IV			
1	1	Introduction to Gene transfer techniques	T1: 166-171
2	1	Microinjection	T1: 166-167
3	1	Biolistic methods	R1: 584-586
4	1	Vector based transfer	R1: 581-583
5	1	Introduction to plant tissue culture	T1: 227-304
6	1	Plant tissue culture-Media composition,	T1: 280-283
7	1	Plant tissue culture-nutrients and growth regulators	T1: 281-283
8	1	Callus culture	T1: 285-286
9	1	Genetic engineering of plants-methodology	T1: 134-139
10	1	Plant transformation with Ti plasmid of Agrobacterium tumifaciens	T1: 136-138
11	1	Herbicide introduction	T1: 327-328

12	1	Production of herbicide resistance plant	T1: 327-328
13	1	Applications of transgenic plants	T2: 194-196
14	1	Revision and Possible QP discussion	
15	1	Revision and Possible QP discussion	
Total No. of Hours planned for Unit IV is 15 hours			
UNIT V			
1	1	Introduction to Genetic engineering of animals	R1: 75-76
2	1	Genetic engineering of animals - Methodology	T2: 39-48
3	1	Production of transgenic mice	R1: 480-482
4	1	Knock out mice	R1: 486-487
5	1	Applications of Transgenic animals	T1: 272-273
6	1	Introduction to Animal biotechnology	T1: 228-229
7	1	Artificial insemination and embryo transfer	T1: 252-256
8	1	In vitro fertilization (IVF)	T1: 256-261
9	1	Introduction to Animal cell culture	T1: 221-223
10	1	Facilities and culture media for animal cell culture	T1: 223-237
11	1	Primary cell culture techniques-cell separation and monolayer culture, Cell lines	T1: 228-237
12	1	Recombinant proteins from cell cultures: interferons, viral vaccines	R1: 196-198
13	1	Gene therapy	R1: 157-169
14	1	Revision and Possible QP discussion	
15	1	Revision and Possible QP discussion	
Total No. of Hours planned for Unit V is 15 hours			

TEXT BOOKS

T1. Dubey, R.C. (2009). *A Textbook of Biotechnology* (19th ed.). New Delhi, S. Chand and Compamy Ltd.

T2. Pandian, T.T., Kandavel, D. (2008). *Textbook of Biotechnology*. New Delhi. I.K. International Publishing House Pvt. Ltd.

REFERNCES

R1. Satyanarayana, U. (2005). *Biotechnology*. (th ed.) Kolkata, Books and Allied (P). Ltd

WEBSITES

W1. http://www.biologydiscussion.com/gene/gene-expression/manipulation-of-gene-expression-in-host-cells-with-diagram/9913

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SYLLABUS

SUBJECT NAME: CORE ELECTIVE II - INTRODUCTION TO BIOTECHNOLOGY

SUB.CODE: 15BCU603A

SEMESTER: VI **CLASS: III B.Sc., BIOCHEMISTRY**

Programme Objectives

To make the student to understand the concept of gene manipulation and gene transfer technologies. To understand the expression systems and method of selection in both plant and animals.

Programme Learning Outcome

Students after completing this programme are able to analyze gene manipulation and gene transfer technologies and also able to work under the expression systems and method of selection in both plant and animals

UNIT -I

Introduction – Basic steps in gene cloning. Vehicles in gene cloning-Plasmids- basic features, size and copy number, classification. Bacteriophage - basic features, life cycle. Cosmids and phagemids. Viral vectors and plant vectors.

UNIT- II

Introduction of DNA into living cells-methods, microinjection, electroporation, shotgun methods. Transformation and transfection in *E.coli* – recombinant selection and screening.
Maximizing the expression of cloned genes in *E.coli*- Promoters, Cassettes and production of fusion protein.

UNIT- III

DNA sequencing-Sanger and Maxim Gilbert method.PCR- techniques and applications.
DNA foot and finger printing- applications. Hybridization probes- radiolabelled and non-radiolabelled.Hybridization techniques- Southern, Northern, Western blotting techniques. Site directed mutagenesis. DNA microarray.

UNITI- IV

Gene transfer techniques- Microinjection, biolistic methods, vector based transfer.Plant tissue culture-Media composition, nutrients and growth regulators, callus culture. Genetic engineering of plants-methodology- plant transformation with Ti plasmidof *Agrobacterium tumifaciens*.

Production of herbicide resistance plant (with reference to glyphosate only). Applications of transgenic plants.

UNIT –V

Genetic engineering of animals – methodology-production of transgenic mice (with reference to insulin only). Knock out mice, Applications of Transgenic animals. Animal biotechnology-Artificial insemination and embryo transfer. *In vitro* fertilization (IVF).

Animal cell culture-Facilities and culture media for animal cell culture. Primary cell culture techniques-cell separation and monolayer culture. Cell lines. Recombinant proteins from cell cultures: interferons, viral vaccines.Gene therapy.

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DEPARTMENT OF BIOCHEMISTRY
III-B.Sc., BIOCHEMISTRY
15BCU603A –CORE ELECTIVE II-INTRODUCTION TO BIOTECHNOLOGY
MULTIPLE CHOICE QUESTIONS

UNIT I

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	The term episome was coined by	Jacob and Wollman	Lederberg	Watson	Collins	**Jacob and Wollman**
2	PBR322 plasmid vectors have	Amp resistance gene	Tet resistance gene	BamHI site	All the above	**All the above**
3	Runaway vectors replicate upto	34˚C	47˚C	94˚C	72˚C	**34˚C**
4	Eukaryotes that habour a plasmid	Fusarium	Saccharromyces cerevisiae	Pencillium	Aspergillus	**Saccharromyces cerevisiae**
5	There are two types of naturally occurring plasmids	Col plasmid	F plasmid	Both a and b	PBR322	**Both a and b**
6	Plasmids are extra chromosomal DNA that vary in size from	1Kb to More than 200kb	2Kb to Morethan 100kb	5Kb to Morethan 200kb	Morethan 300kb	**1Kb to More than 200kb**
7	Word Plasmid was coined by	Maccarthy	Avery	Lederberg	Smith	**Lederberg**
8	PBR322 Plasmid carries	PMB1 replicon	P15A replicon	ColE1 replicon	PkN402	**PMB1 replicon**
9	The copy number of plasmid was controlled by	Host chromosome	Regulatory genes	Motar genes	Replicons	**Replicons**
10	Plasmids are commonly found in	Algae	Virus	Protozoa	Prokaryotes	**Prokaryotes**
11	Conjugation is biological phenomena observed in the bacterial genetics directly by	Functional genes	Transfer genes and Mobilizing genes	Nick genes	Basis of mobilizing genes	**Transfer genes and Mobilizing genes**
12	The bacteria associated with the discovery of R plasmids	Bacillus	Pseudomonas	Shigella	Salmonella	**Shigella**
13	Non conjugative plasmids have	tra+/ mob+ genes	tra-/ mob- genes	tra-/ mob+ genes	mob+/ mob+ genes	**tra-/ mob+ genes**
14	Plasmids carrying genes which is responsible for antibiotic resistance known as	R plasmids	Canser inducing plasmid	Col E1 plasmid	F plasmid	**R plasmids**
15	Plasmid contain autonomous replication (rep) includes	Origin of repliation	Selectable marker	Copy number	All the above	**All the above**
16	DNA replication during lytic growth required for the lysis of cellular membrane	S and R	R and T	S and Q	Q and U	**S and R**
17	Vectors, which contains a unique site (x) for the insertion of foreign DNA have been designated as	Expression vector	Replacement vector	Insertional vector	Shuttle vector	**Insertional vector**
18	λgt10 carry up to	8 Kb of new DNA	10kb new DNA	15kb new DNA	20 kb new DNA	**10kb new DNA**
19	λgt10 E CORI site is located in	R genes	C 1 genes	b 2 genes	D genes	**C 1 genes**
20	E.CORI used in *E.coli* how many fragments	6	7	8	9	**8**
21	One gene important for ampicillin resistant in PBR322 is	D galactose gene	β –galactose gene	β- lactamase gene	β- Lactase gene	**β- lactamase gene**
22	PBR 322 vectors are restricted to	Gram positive bacteria	Gram negative bacteria	Archae bacteria	Eubacteria.	**Gram negative bacteria**
23	Lambda DNA has a genetic material as	ds DNA	Ss DNA	ds RNA	ss RNA	**Ss DNA**
24	Lambda genome has a length of	48502bp	33402bp	40000bp	50,435bp	**48502bp**
25	The non essential region of lambda phage is	int,xis,mob	int,xis,att	tra,mob,xis	B2 region	**B2 region**
26	The plasmid that maintains low copy number in a cell	Stringent plasmid	Relaxed plasmid	Conjugative plasmids	None of the above	**Stringent plasmid**
27	The genes for drug resistance is initially coded on	PBR322	Transposons	Yeast plasmid	None of the above	**PBR322**
28	PBR322 composed of	3 section	2 section	1 section	5 section	**3 section**
29	Tn3 from transposon for PBR322 have	Tetracyclin resistant	Streptomycin resistant	Ampicillin resistant	Chloramphenicol resistant	**Ampicillin resistant**

30	The best studied Col plasmid is	ColA	ColB	ColE	ColD	**ColE**
31	PBR322 is a	Natural plasmid	Artificial plasmid	Neutral plasmid	Nature plasmid	**Artificial plasmid**
32	λ EMBL4 carries up to	10 Kb	20kb	15kb	25 kb	**10 Kb**
33	λ EMBL4 selected on the basis of	Size / Spiphenotype	Size / X gal Phenotype	Size / Non recombinants	None.	**Size / Spiphenotype**
34	The viral genome of SV 40is	5243 bp	5842bp	3483bp	2800bp	**5243 bp**
35	SV40 DNA is isolated from virus particles in the form of	Super helical DNA	Double Stranded Super helix DNA	Both a and b	Inner DNA	**Both a and b**
36	Vectors, which contains two restriction sites for the insertion of foreign DNA have been designated as	Expression vector	Replacement vector	Insertional vector	Shuttle vector	**Replacement vector**
37	The first step I to create recombinant AcMNPV is to generate	Transfer vector	Helper vector	Modified vector	Initiator sequence	**Transfer vector**
38	Copy number are either strictly controlled by correlating with the number of	Plasmid DNA	Chromosomal DNA	Insertional DNA	None of the above	**Chromosomal DNA**
39	During infection -------- forms of virons produced	one form	Two form	Three form	Fiveform	**Two form**
40	In second form the protein of matrix is called as ----------	Monohedrin	Polyhedrin	Dihedrin	Trihedrin	**Polyhedrin**
41	Agrobacterium tumefaciens infects	Monocotyledon Palnts	Dicotyledon Plants	Both a and b	Polycotyledon Plants	**Dicotyledon Plants**
42	Ti Plasmids are	Transgenic plasmids	Tumour inducing plasmids	Inhibiting plasmids	Haemorhageic plasmids	**Tumour inducing plasmids**
43	In reterovires gag codes for	Reverse transcriptase	Polymerase	Synthase	Envelop proteins	**Reverse transcriptase**
44	In reterovirus pol codes for	Reverse transcriptase	Polymerase	Synthase	Envelop proteins	**Polymerase**
45	In reterovirus env codes for	Revese transcriptase	Polymerase	Synthase	Envelope proteins	**Envelope proteins**
46	Early region codes for two partially overlapping genes, which direct the synthesis of	T –Antigen	t-antigen	R-antigen	r –antigen	**T –Antigen**
47	The function of T antigen	Initiation DNA replication	Control of its own transcription	Both a and b	Control of its own translation	**Initiation DNA replication**
48	Which organism is called as "Super Bug"	Escherichia coli	Bacillus spp	Pseudomonas putida	Streptococcus albicans	**Pseudomonas putida**
49	Vir E codes for which protein that binds to T-Dna during transfer	Competence factors	SSB proteins	DSB proteins	Tyrosine	**SSB proteins**
50	PH V 33 Multiply both in	E.Coli and Bacillus	Bacillus and Streptomyces	Bacillus and Yeast	Bacillus and Pseudomonas	**E.Coli and Bacillus**
51	The most common type of vector are ---------- that have been genetically altered to carry normal human DNA	PBR322	Cosmids	Viruses	Yeast	**Viruses**
52	The marker gene present in PBR322 is	Ampr	Kamr	Camr	Metr	**Amp**r
53	Marker inactivation of antibiotics can be studied by	Activity of antibiotics	Resitance of antibiotics	Both resistance and activity	None of the above	**Resitance of antibiotics**
54	Which type of restriction enzymes is used in gene cloning?	Type I	Type II	Type III	All the above	**All the above**
55	The extra chromosomal DNA molecule that can replicate independently inside the host cell	Plasmids	Chloroplasts	Mitochondria	Genes	**Plasmids**
56	Puc8 vector is ---------- selection vector	Amp selection vector	Kan selection vector	Lac selection vector	Spi selection vector	**Lac selection vector**
57	In PBR322 the word BR stands for	Berger	Bunsen rodergh	Beep run	Boliver and Rodriguez	**Boliver and Rodriguez**
58	PBR322 was developed in	1960	1980	1970	1990	**1970**
59	PGEM vector have	SP6 promoter	T7 Promoter	Both SP6 and T7	T8 Promoter	**Both SP6 and T7**
60	---------- is produced from E.coli	F plasmid and Col E plasmid	Ti plasmids	Degradative plasmids	None of the above	**F plasmid and Col E plasmid**

UNIT-I
SYLLABUS

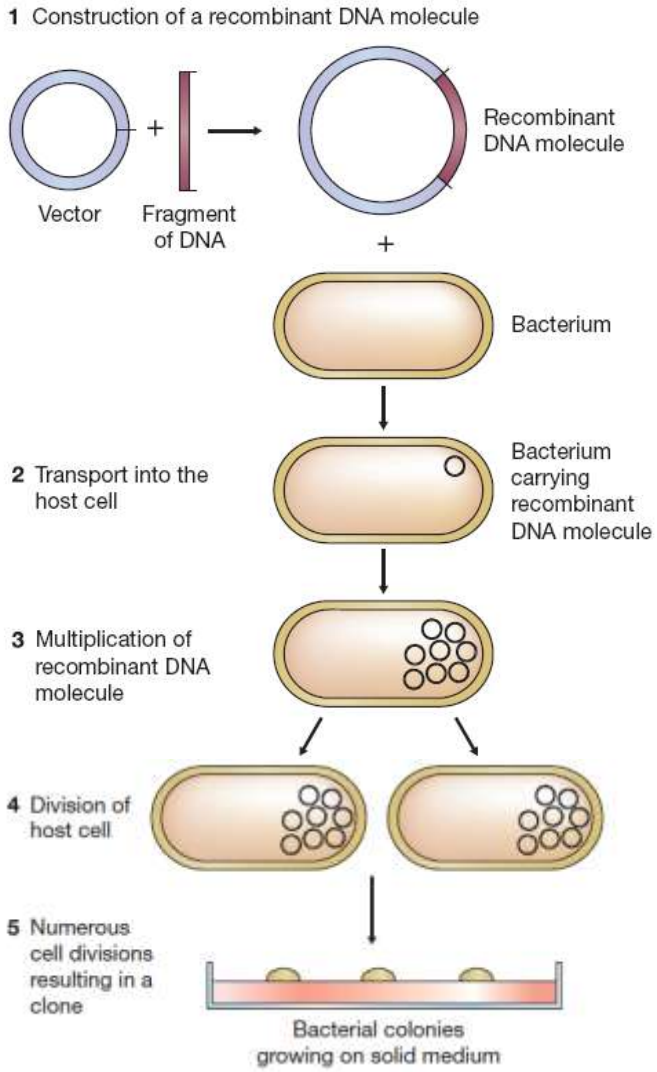
Introduction – Basic steps in gene cloning. Vehicles in gene cloning-Plasmids- basic features, size and copy number, classification. Bacteriophage - basic features, life cycle. Cosmids and phagemids. Viral vectors and plant vectors.

Introduction - Basic steps in gene cloning

The goal of rDNA is gene cloning to generate large amounts of pure DNA that can be manipulated and studied. The following are the basic steps involved in the process of the rDNA technique for gene cloning:

The steps in a gene cloning experiment:

- A fragment of DNA, containing the gene to be cloned, is inserted into a circular DNA molecule called a vector, to produce a recombinant DNA molecule.
- The vector transports the gene into a host cell, which is usually a bacterium, although other types of living cell can be used.
- Within the host cell the vector multiplies, producing numerous identical copies, not only of itself but also of the gene that it carries.
- When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place.
- After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.



Vehicles in gene cloning

Vectors: The Vehicle for Cloning

One of the major applications of rDNA experiments is the gene cloning or DNA cloning. Vectors are another major component required to make an rDNA molecule for gene cloning. Vectors act as a vehicle for carrying foreign DNA into a host cell for multiplication. Usually small circular DNA molecules of bacterial origin are used as cloning vectors. A DNA molecule should possess the following essential characteristics to act as a cloning vector:

Origin of Replication

It is required for autonomous replication of the plasmid using the host's replication machinery. Almost all commonly used plasmids are based on the ColE1 origin of replication (ori). Naturally occurring origins of replication are negatively regulated to keep the copy number

down (typically five to ten copies per cell) to reduce the load on the host's replication machinery. While a high copy number is disadvantageous in a natural system, it is a desirable feature in a cloning vector— since the whole idea of gene cloning is to easily isolate substantial quantities of a particular DNA sequence. Modern plasmid vectors are therefore often called 'runaway replicons' and are present at 100 to 1,000 copies per cell.

Selectable Markers

Selectable markers are essential for the identification of bacteria containing recombinant plasmids. Selection can be divided into two types—**positive selection** and **negative selection**.

- **Positive selection** is used to identify bacteria that contain plasmids. The most common markers used for positive selection are the antibiotic resistance genes carried by the original R factors. While many antibiotics and resistance genes are available, the commonly used ones fall into two general classes: **Antibiotics affecting cell wall synthesis** and **Antibiotics affecting translation.** Ampicillin is a beta-lactam-based antibiotic that acts by inhibiting the synthesis of the bacterial peptidoglycan cell wall. Sensitive bacteria are not actively 'killed,' but on cell division are unable to synthesize the cell wall and suffer from osmotic lysis. The enzyme beta-lactamase is secreted into the periplasmic space where it breaks down the antibiotic, allowing cell wall synthesis to proceed. The antibiotics tetracycline, kanamycin, and chloramphenicol all act by inhibiting translation. The covalent modification (phosphorylation, acetylation) of these antibiotics blocks their interaction with the translation apparatus. Positive selection is particularly important when introducing plasmids into bacteria by transformation. At best, only about 1 in 10,000 bacteria picks up a plasmid that carries the antibiotic resistance. A strong positive selection system is essential to eliminate the 9,999 bacteria that did not pick up a plasmid from the one that did. By plating the transformation products directly on antibiotic plates, all untransformed bacteria die and only those containing the plasmid (and antibiotic-resistance marker) grow to form colonies.
- A second selection system is necessary to distinguish between plasmids that are merely recircularized from those that carry a foreign DNA insert. Usually, a negative selection method is used. In order to identify those plasmids carrying a foreign DNA fragment, the site of insertion is chosen such that the insertion disrupts a selectable marker—a phenomenon known as **insertional inactivation**. It can be the insertional inactivation of an antibiotic resistance gene or enzymes such as β-galactosidase, a product of *Lac* Z gene of lac operon. The insertion of the foreignDNA into the vector will disrupt the expression of these genes and will produce a color difference for the colony from that of cells with intact vectors.

Multiple Cloning Sites (MCS) or Polylinker

A vector should have a site specific for cloning the foreign DNA fragment provided with one restriction site for most of the commonly used unique restriction endonucleases. All these unique restriction sites are grouped together in a small region of the vector known as the **multiple cloning site (MCS)** or the **polylinker**. The presence of unique restriction sites at the MCS gives flexibility in the choice of restriction enzymes.

Small Size

Relatively small vectors are more desirable because they increase the transformation efficiency and are easy to manipulate. Small size also helps purification procedures to obtain intact plasmids. A large number of vectors with the above characteristics have been developed.
There are six different types of cloning vectors commonly used in recombinant DNA experiments. They are the following:

- Plasmid-cloning vectors
- Bacteriophage cloning vectors
- Cosmid-cloning vectors
- Yeast artificial chromosomes (YACs)
- Bacterial artificial chromosomes (BACs)
- Animal and plant vectors (Shuttle vectors)

Plasmids- basic features, size and copy number, classification

Plasmids are extra chromosomal circular double stranded DNA. These vectors are derived from bacterial plasmids and are the most widely used, versatile, and easily manipulated ones. Plasmid are used as cloning vectors, include ori site or origin of replication, needed for replication in the bacterial cells. For an example E.coli plasmid vector, should have following features:
a. Ori site for replication.
b. Selectable marker genes, such as antibiotic resistance gene
c. Unique restriction sites, so that the restriction enzymes can be used to cut the plasmid and DNA of interest can be inserted into the plasmid.

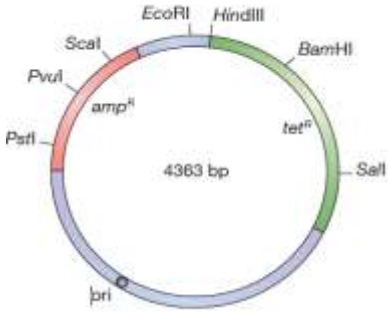
ColE1 of *E. coli* is an example of a naturally occurring plasmid. The *ori* in almost all plasmid vectors is that of ColE1. The following are some of the examples of plasmid-cloning vectors.

a)pBR322 Vectors

The name "pBR322" conforms with the standard rules for vector nomenclature:
"p" indicates that this is indeed a plasmid.
"BR" identifies the laboratory in which the vector was originally constructed
(BR stands for Bolivar and Rodriguez, the two researchers who developed pBR322).
"322" distinguishes this plasmid from others developed in the same laboratory
(there are also plasmids called pBR325, pBR327, pBR328, etc.).

This was the first widely used, purpose built plasmid vector. It has a number of useful features:
Origin of replication. It carries a fragment of the plasmid pMB1 that acts as an origin for DNA replication and thus ensures multiplication of the vector.
Size. It is relatively small at 4,363 bp. This is important because transformation efficiency is inversely proportional to size and above 10 kbp is very low. Thus, there is 'room' in pBR322 for an insert of at least six kbp.
Copy number. Reasonably high copy number (~15 copies per cell), which can be increased 200-fold by treatment with a protein-synthesis inhibitor—chloramphenicol amplification.
Selectable marker. It carries two antibiotic resistance genes—ampicillin and tetracycline.

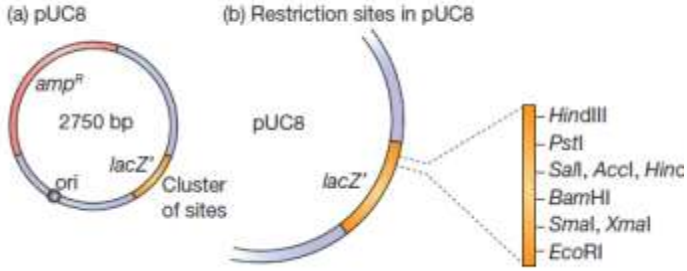
Cloning sites. It carries a number of unique restriction sites. Some of these are located in one of the antibiotic resistance genes (e.g., sites for *Pst* I, *Pvu* I, and *Sac* I are found in *Ampr* and *Bam*HI and *Hind* III in *Tetr*). Cloning into one of these sites inactivates the gene allowing recombinants to be differentiated from non-recombinants known as **insertional inactivation.**



Gene map of pBR322

b)pUC Series Vectors

Another popular plasmid vector is the pUC series, which is extensively used as cloning as well as expression vector. These vectors have three important additional features compared to pBR322.



pUC8 vector

High copy number. A mutation within the origin of replication produces 500to 600 copies of the plasmid per cell without amplification.

Blue-white screening. This is a special form of insertional activation that can be used during the primary selection of transformants, rather than requiring a second round of screening. It utilizes the N-terminal portion of the *E. coli* beta-galactosidase—the product of lac Z gene (alpha-peptide) encoded by the vector in a form of intermolecular complementation—that restores betagalactosidase activity to a defective enzyme (omega-peptide) encoded by the host. If a chromogenic substrate (X-gal) and a beta-galactosidase inducer (IPTG) are included in the plates on which the primary transformants are selected, non-recombinant molecules will catabolize the colorless substrate to give blue colonies.

A synthetic polylinker. This is a piece of manmade DNA that contains several unique restriction sites. It has been inserted within the portion of the vector encoding the beta-galactosidase alpha-peptide in such a way that it does not affect its expression. However,

inserting a foreign DNA fragment into any one of the polylinker restriction sites almost invariably disrupts the enzyme activity. Thus, recombinant colonies remain white but non-recombinants turn blue.

Expression Vectors

In most of the cases the main purpose of the rDNA experiment and cloning is to sufficiently multiply or amplify the inserted DNA fragment. But sometimes the aim of the process will be to produce large quantities of protein encoded by the inserted gene. This can be accomplished by incorporating the necessary regulatory elements along with the gene in the vector. Such vectors should be provided with signals necessary for initiation and termination of transcription such as suitable promoters and terminator sequences; and signals for translation initiation such as a start codon and a ribosome binding site into the vector upstream to the multiple cloning site (MCS). These vectors, which have the regulatory elements and other machinery for the expression of the cloned gene, can be used for the production of recombinant proteins and are known as **expression vectors**. Vectors of the pUC series are example of expression vectors.

pUC19 is an example for an E.coli cloning vector. pUC19 is basically 2,686bp in length. The features of this pUC19 plasmids are as follows:

a. High copy number, as high as nearly hundred copies per bacterial cell. This helps in getting good yield of cloned DNA in short duration.

b. It also has got selectable marker as ampicillin resistance gene.

c. This plasmid also got a cluster of unique restriction sites known as polylinkers or in other words this plasmid has got multiple cloning sites.

d. The polylinkers or multiple cloning sites are also a part of lacZ (galactosidase) gene. That is pUC19 plasmid will complement a β lacZ E.coli allowing it to become lacZ positive. When DNA of interest is cloned into the polylinkers lacZ is disrupted, this prevents the complementation from occurring.

e. A chromogenic analog of lactose known as X-gal, turns blue in presence of galactosidase, and remains white in β absence; therefore blue white colonies can be used as screening method to identify the bacterial colonies which contain recombinant plasmid.

DNA of interest is inserted into a cloning vector using restriction enzyme and then ligating it with enzyme ligase.

a. pUC19 plasmid vector can be cut using restriction enzyme that has got a unique site in the polylinker region.

b. DNA of interest is also cut using the same restriction enzyme.

c. DNA of interest and pUC19 DNA are mixed and allowed for random joining of fragments.

d. Resulting plasmids then transformed into E.coli cells either by chemical treatment method or by electroporation method. Then these bacterial cells are grown on media contain ampicillin and Xgal.

e. Ampicillin resistance is resulted from pUC19 sequence, Blue colonies are resulted from the rejoined plasmids, and white colonies will result from transformed bacterial cells with recombinant plasmids.

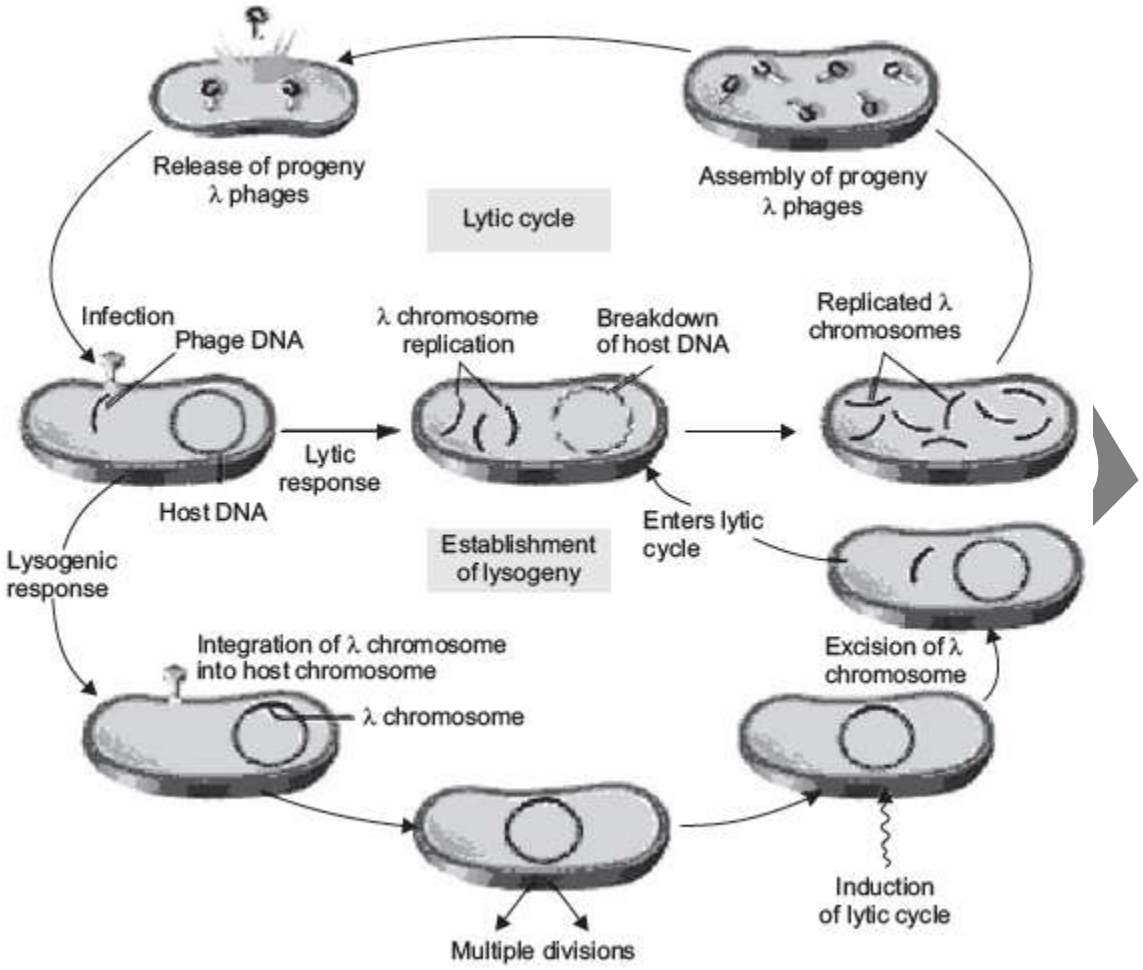
Many different types of cloning vectors are designed in the laboratory and they are commercially available for use. These vectors have different array of unique restriction sites in the polylinker site. Many plasmid cloning vectors are designed and available for many prokaryotic organisms as well as for eukaryotic organisms. But the plasmid cloning vectors which carry more than 5 to 10 kb gene of interest are often unstable. Therefore size of the gene of interest is limited in plasmid cloning vectors.

Bacteriophage - basic features, life cycle

These are derived from viruses that specifically infect bacteria. Such viruses usually contain a comparatively small DNA genome surrounded by a protein coat. The viruses that infect bacteria are called **bacteriophages**. Those phages, which most frequently infect *E. coli*, are used for cloning purposes. Bacteriophages infect bacterial cells by injecting their DNA into the host cytoplasm. This DNA in the host cell selectively replicates and expresses the proteins required for the assembly of new phage particles. These processes result in the production of a large number of phages, which break the cells with a process known as the **lytic cycle**. These new viral particles will re-infect the neighboring cells and the cycle continues. In some cases the phage DNA, after entering into the host cell, is integrated along with the host genome and is separated at a later stage and starts the lytic cycle as explained above. The lytic cycle and phage multiplication are shown in Figure.

The natural way of transferring the viral DNA into a specific bacterial host has attracted scientists and they have modified these viral genome DNA to use as vectors for gene cloning. Bacteriophage lambda (λphage) and M13 are extensively modified for the development of a phage-based cloning vector.

There are many versions or types of bacteriopage λ used as cloning vectors. For example sequence of the gene responsible for the lysogeny is removed, so that only one type that is lytic infection is possible in this bacteriophage λ vector. The central region of the λ chromosome is used for inserting the gene of interest. Gene of interest is inserted using restriction digestion and also DNA ligation method.



The bacteriophage infection and life cycle—lysogeny and lytic cycle.

When this ligated DNA is mixed with phage proteins, this leads to the assembling of phage head and DNA is also packed to form virus particles. Only viral particles with both the phage chromosome arms and also with proper central segment (3752kb) are able to replicate by infecting the E.coli bacterial cell. Progeny phages contain DNA of interest; hence provide a large quantity of DNA of interest. Many types of phage cloning vectors are available with varying features like an expanded array of restriction sites and much more

a)ƛ Phage-cloning Vectors

These are the engineered version of Lambda bacteriophage that infects *E. coli*. This vector has a double-stranded linear DNA genome having a size of 48,514 bp (48.5 kb). About 12 bases at the ends of this DNA molecule are unpaired and complementary and therefore are sticky or cohesive and known as **cos sites (cohesive end sites).** These cohesive sites are very essential for

packaging the DNA into the viral particle during the lytic cycle. But a major portion of the central region of the viral genome DNA is not required for the infection and lytic cycle in *E. coli* cells. The λphage vector is designed in such a way that the central region of the λchromosome (linear) is cut with a restriction enzyme and is replaced with the foreign DNA digested with the same restriction enzyme. This recombinant DNA is packaged in phage heads to form virus particles (Figure). Phages with both ends of the λ chromosome and a 37 to 52 kb insert replicate by infecting *e. coli*. Phages replicate using *E. coli* and the lytic cycle (Figure) and produce large quantities of 37 to 52 kb cloned DNA. A large number of unique restriction sites are also available here.

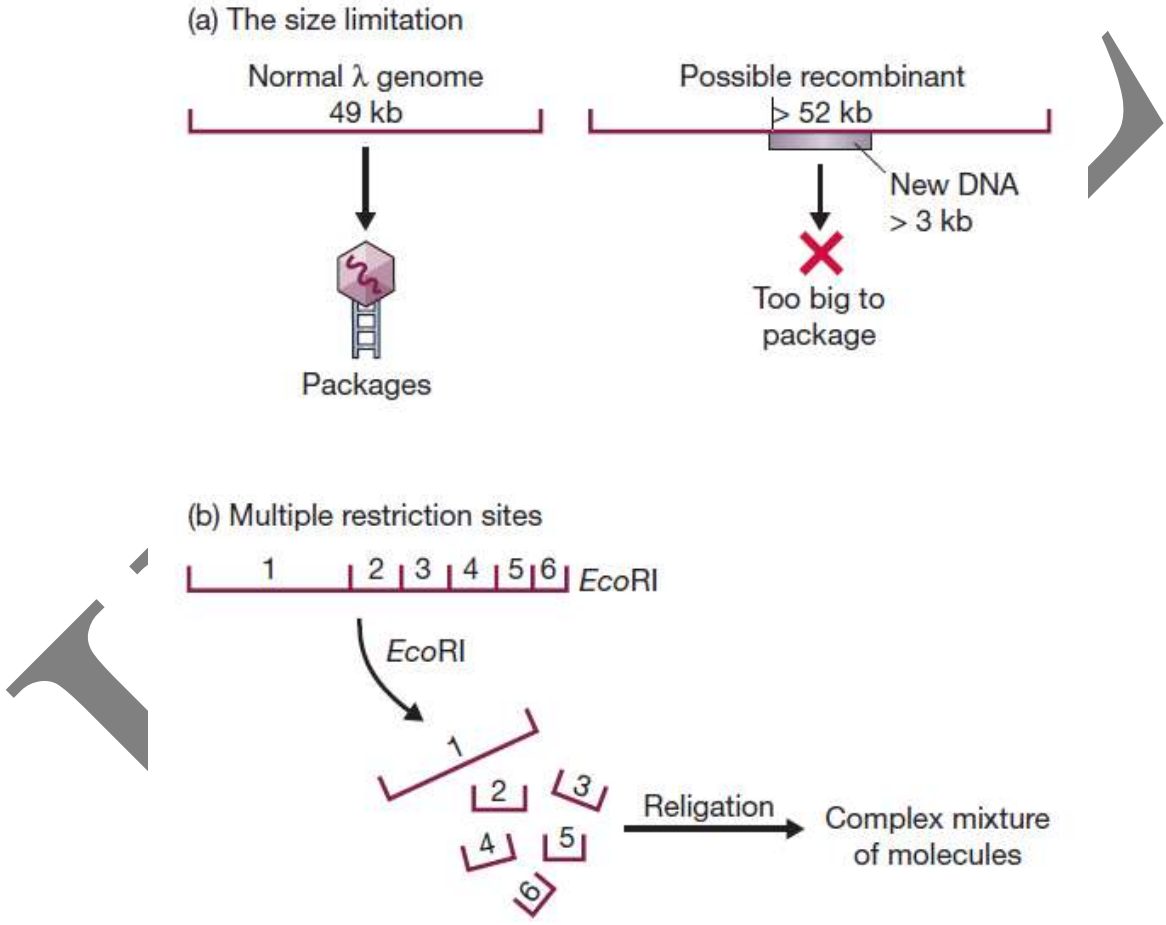


Fig: The two problems that had to be solved before cloning vectors could be developed. (a) The size limitation placed on the λ genome by the need to package it into the phage head. (b) λ DNA has multiple recognition sites for almost all restriction endonucleases.

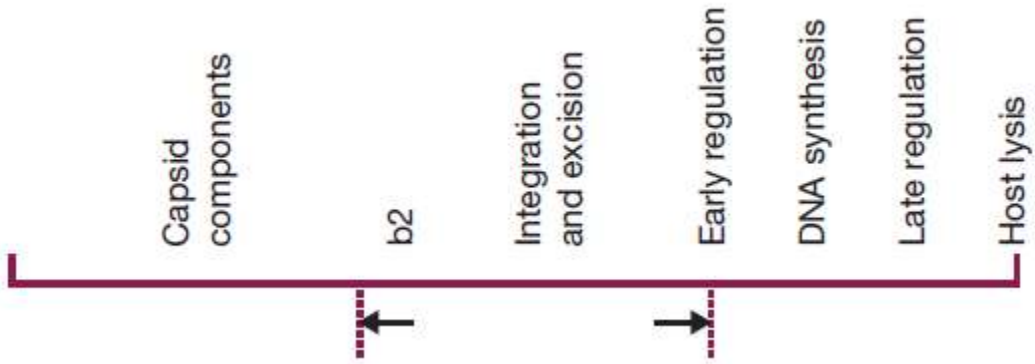


Fig: The λ genetic map, showing the position of the main non-essential region that can be deleted without affecting the ability of the phage to follow the lytic infection cycle. There are other, much shorter non-essential regions in other parts of the genome.

Insertion and replacement vectors for bacteriophages

Once the problems posed by packaging constraints and by the multiple restriction sites had been solved, the way was open for the development of different types of e-based cloning vectors. The first two classes of vector to be produced were λ **insertion** and λ**replacement** (or substitution) vectors.

a)Insertion vectors

With an insertion vector (Figure a), 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, of course, on the extent to which the non-essential region has been deleted. Two popular insertion vectors are:

λgt10 (Figure 6.12b), which can carry up to 8 kb of new DNA, inserted into a unique *Eco*RI site located in the *c*I gene. Insertional inactivation of this gene means that recombinants are distinguished as clear rather than turbid plaques .

λZAPII (Figure 6.12c), with which insertion of up to 10 kb DNA into any of 6 restriction sites within a polylinker inactivates the *lacZ*2 gene carried by the vector. Recombinants give clear rather than blue plaques on X-gal agar.

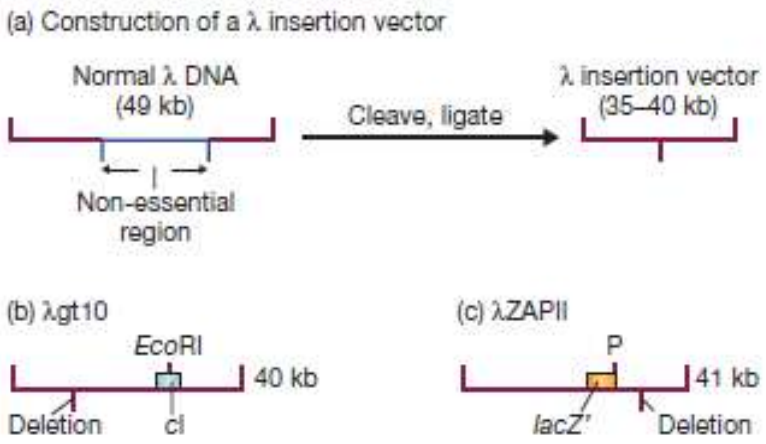


Fig: λ insertion vectors. P = polylinker in the *lacZ*2 gene of ⌊ZAPII, containing unique restriction sites for *Sac*I, *Not*I, *Xba*I, *Spe*I, *Eco*RI, and *Xho*I

b) Replacement vectors

A e replacement vector has two recognition sites for the restriction endonuclease used for cloning. These sites flank a segment of DNA that is replaced by the DNA to be cloned (Figure). Often the replaceable fragment (or **"stuffer fragment"** in cloning jargon) carries additional restriction sites that can be used to cut it up into small pieces, so that its own re-insertion during a cloning experiment is very unlikely. Replacement vectors are generally designed to carry larger pieces of DNA than insertion vectors can handle. Recombinant selection is often on the basis of size, with non-recombinant vectors being too small to be packaged into e phage heads

An example of a replacement vectors is:

λ**EMBL4** (Figure b) can carry up to 20 kb of inserted DNA by replacing a segment flanked by pairs of *Eco*RI, *Bam*HI, and *Sal*I sites. Any of these three restriction endonucleases can be used to remove the stuffer fragment, so DNA fragments with a variety of sticky ends can be cloned. Recombinant selection with eEMBL4 can be on the basis of size, or can utilize the Spi phenotype .

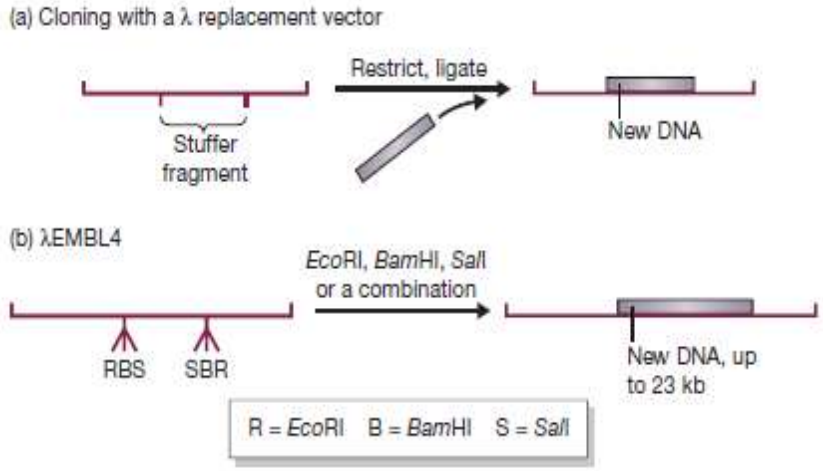


Fig: λ replacement vectors. (a) Cloning with a λ replacement vector. (b) Cloning with λ EMBL4.

b)M13-based Cloning Vectors

The M13 family of vectors is derived from bacteriophage M13. This is a malespecific (infects *e. coli* having *f. pili*), lysogenic filamentous phage with a **circular single-stranded DNA** genome about 6,407 bp (6.4 kb) in length. On infection, this molecule is transferred to *E coli* and converted into the double-stranded replicative form (RF). The replication continues and when there are more than 100 copies of DNA in the cell, the DNA replications become asymmetric and produce copies of the original single-stranded molecule, which are packaged into infective particles and extruded from the cell. The host is never lysed but continues to grow throughout the infection, although at a significantly reduced rate. This difference in growth rate between infected and uninfected cells produces characteristic 'plaques' when M13 phages are plated on a suitable host.

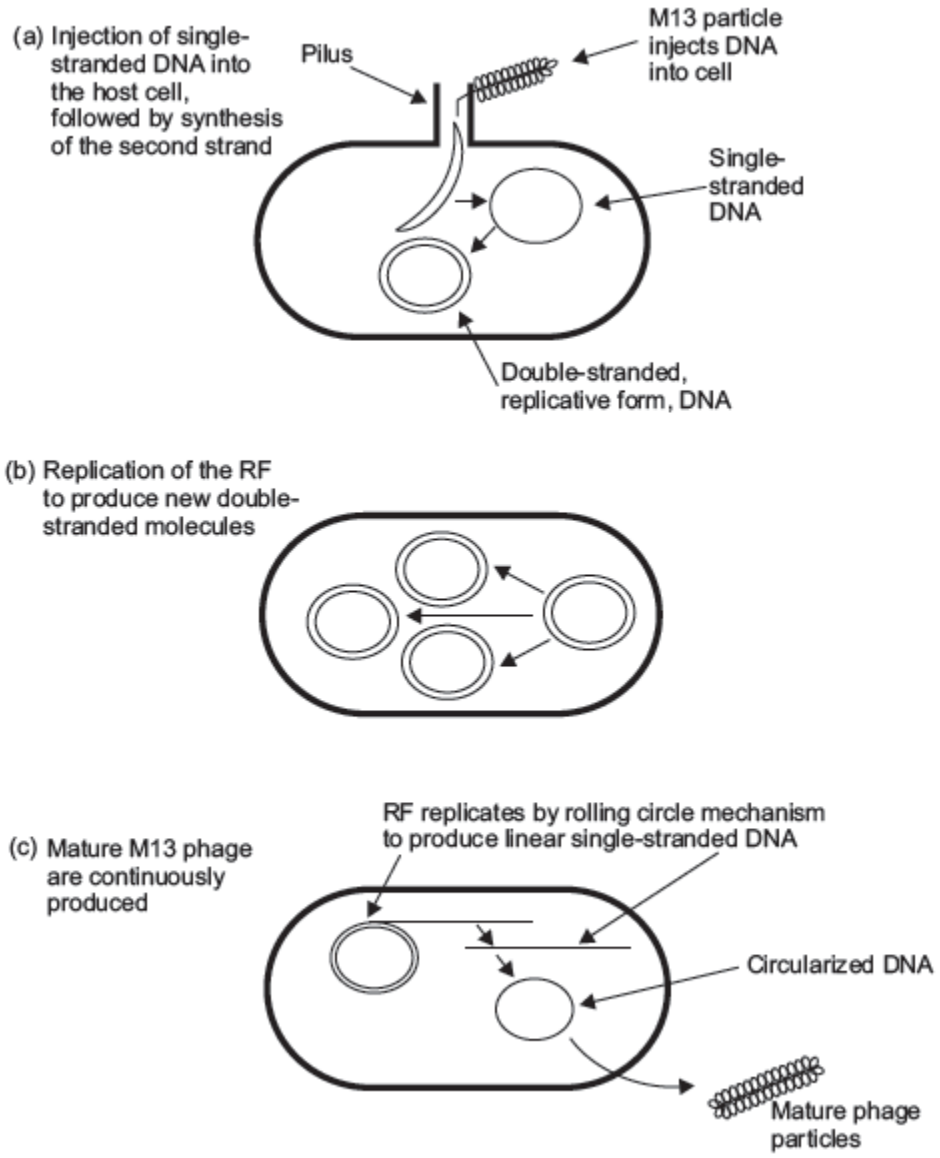


FIGURE Infection and life cycle of M13 phage.

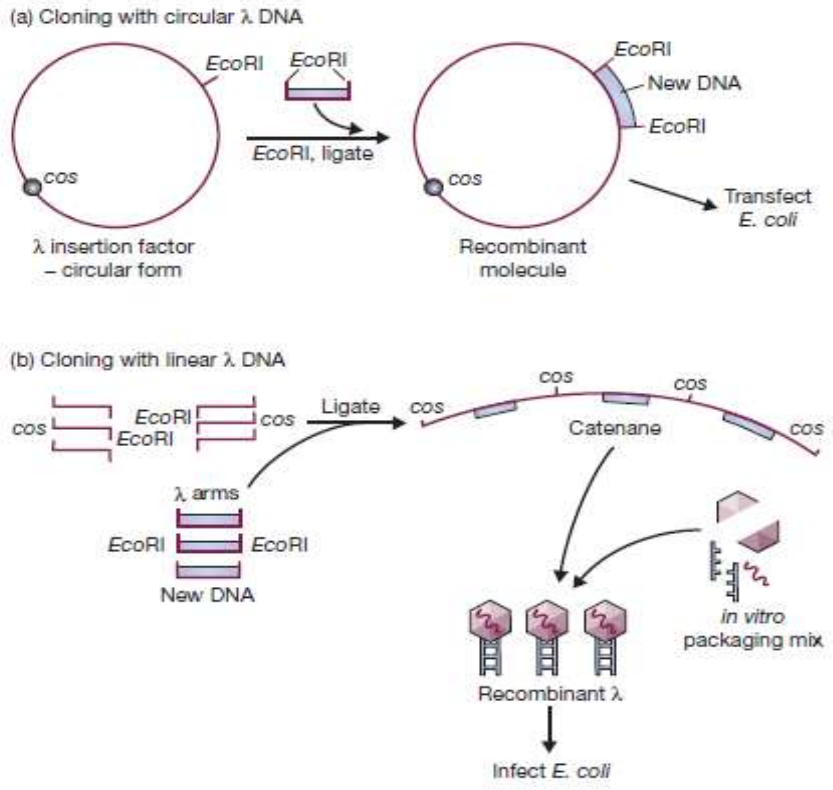
The advantages of M13-based vectors are that they contain the same polylinker and alpha-peptide fragments as the pUC series and recombinants can be selected by the blue → white color test. The RF form of M13 vectors can be isolated by standard plasmid DNA-preparation procedures, and foreign DNA can be inserted into them as if they were conventional plasmids. The size of the genome is below 10 kb and so is easy to handle.

The specific use of M13 is as an aid to DNA sequencing. Once cloned into M13, large amounts of the single-stranded form of any given fragment can be easily isolated from the mature phage that is extruded from infected cells. This is an ideal template for a dideoxy-sequencing reaction.

If an oligonucleotide complementary to the region just downstream of the polylinker is used as the primer for the dideoxy reaction, the cloned DNA fragment can be sequenced. With this method it is possible to determine the sequence of any DNA fragment that has been cloned into M13.

Cloning experiments with insertion or replacement vectors

A cloning experiment with a e vector can proceed along the same lines as with a plasmid vector—the e molecules are restricted, new DNA is added, the mixture is ligated, and the resulting molecules used to transfect a competent *E. coli* host (Figure). This type of experiment requires that the vector be in its circular form, with the *cos* sites hydrogen bonded to each other. Although satisfactory for many purposes, a procedure based on transfection is not particularly efficient. A greater number of recombinants will be obtained if one or two refinements are introduced. The first is to use the linear form of the vector. When the linear form of the vector is digested with the relevant restriction endonuclease, the left and right arms are released as separate fragments. A recombinant molecule can be constructed by mixing together the DNA to be cloned with the vector arms (Figure 6.14b). Ligation results in several molecular arrangements, including catenanes comprising left arm–DNA–right arm repeated many times (Figure 6.14b). If the inserted DNA is the correct size, then the *cos* sites that separate these structures will be the right distance apart for *in vitro* packaging (p. 81). Recombinant phage are therefore produced in the test tube and canbe used to infect an *E. coli* culture. This strategy, in particular the use of *in vitro* packaging, results in a large number of recombinant plaques.

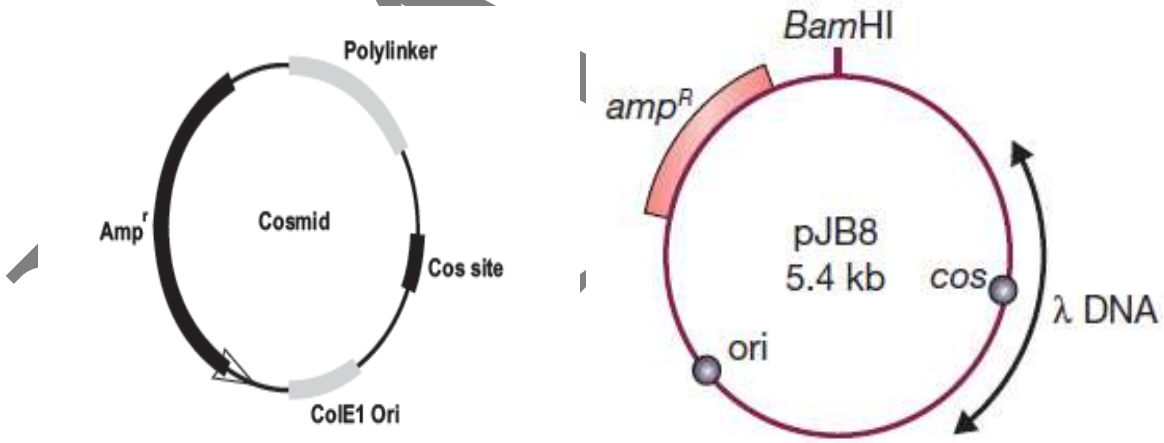


Different strategies for cloning with a λ vector. (a) Using the circular form of λ as a plasmid. (b) Using left and right arms of the λ genome, plus *in vitro* packaging, to achieve a greater number of recombinant plaques

Cosmids

Cosmid-cloning vectors were among the first large insert cloning vehicles developed. They were constructed by certain features of plasmid and the *cos* site (which allows the target DNA to be inserted) of ƛ phage. A cosmid can be defined as a plasmid that contains a *cos* site from the lambda phage genome. The vector replicates as a plasmid (it contains a ColE1 origin of replication), and uses AmpR for positive selection and employs lambda phage packaging to select for recombinant plasmids carrying foreign DNA inserts up to 45 kb in size.

The simplest cosmid vector has a ColE1 origin of replication, selectable markers including the antibiotic-resistance gene and β-galactosidase gene (a part of *lac Z* gene), and suitable polylinker sites and lambda cos site (Figure). Ligation of the cosmid vector and foreign DNA fragments of sizes **upto 45 kb** is similar to ligation into a lambda substitution vector. The desired ligation product is a concatemer of 45 kb foreign DNA fragment and five kb cosmid-vector sequences. This concatemer is then packaged into viral particles (remember, packaging is cos site to cos site) and these are used to infect *E. coli* where the cosmid vector replicates using the ColE1 origin of replication (*ori*). Phage packaging serves only to select for recombinant molecules and to transfer these long DNA molecules (50 kb total) into the bacterial host (50 kb fragments transform very inefficiently while phage infection is very efficient).



A simplest form of cosmid

The following are some advantages of cosmids:

- High transformation efficiency.
- The cosmid vector can carry up to 45 kb whereas plasmid and □ phage vectors are limited to 25 kb.

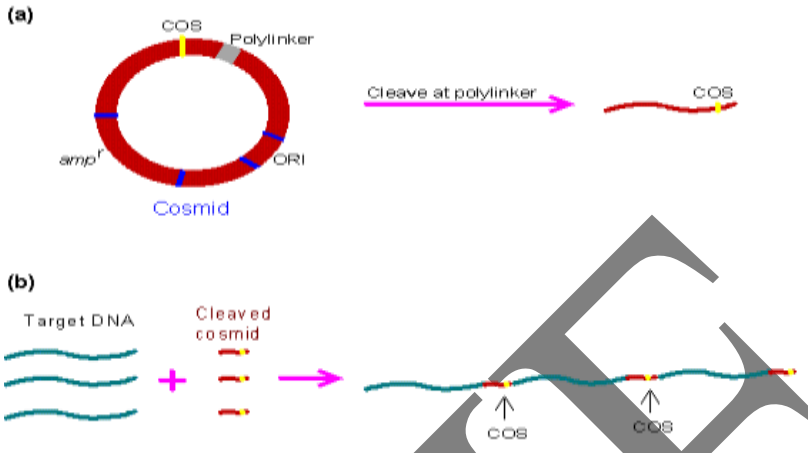
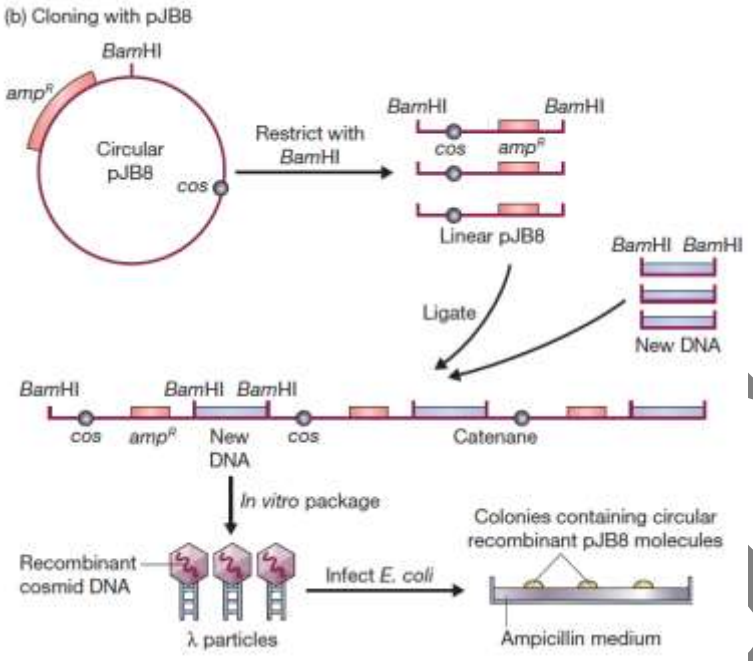


Figure. Cloning by using cosmid vectors. (a) In addition to *amp*^{r}, ORI, and polylinker as in the plasmid vector, the cosmid vector also contains a COS site. (b) After cosmid vectors are cleaved with restriction enzyme, they are ligated with DNA fragments. The subsequent assembly and transformation steps are the same as cloning with phages

A cloning experiment with a cosmid is carried out as follows

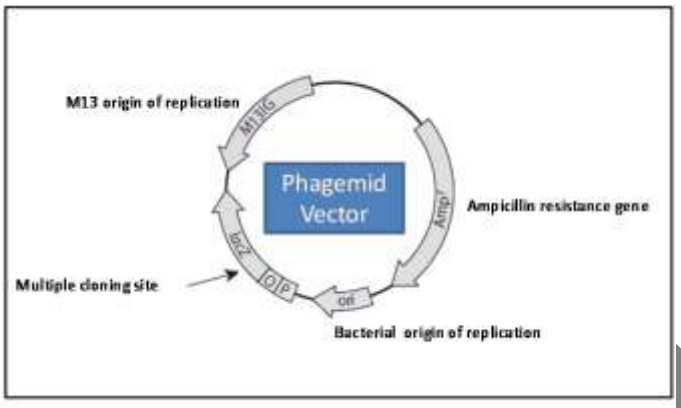
The cosmid is opened at its unique restriction site and new DNA fragments inserted. These fragments are usually produced by partial digestion with a restriction endonuclease, as total digestion almost invariably results in agments that are too small to be cloned with a cosmid. Ligation is carried out so that catenanes are formed. Providing the inserted DNA is the right size, *in vitro* packaging cleaves the *cos* sites and places the recombinant cosmids in mature phage particles. These e phage are then used to infect an *E. coli* culture, though of course plaques are not formed. Instead, infected cells are plated onto a selective medium and antibiotic-resistant colonies are grown. All colonies are recombinants, as non-recombinant linear cosmids are too small to be packaged into e heads.

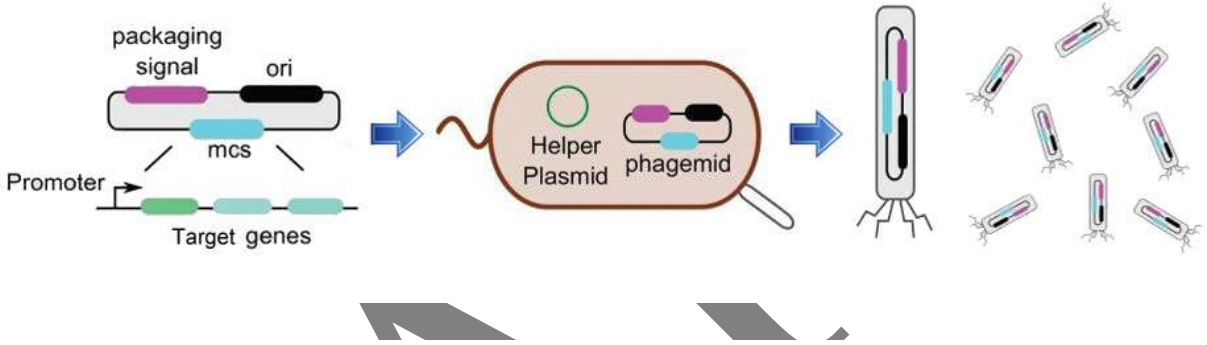


A typical cosmid and the way it is used to clone long fragments of DNA

Phagemids

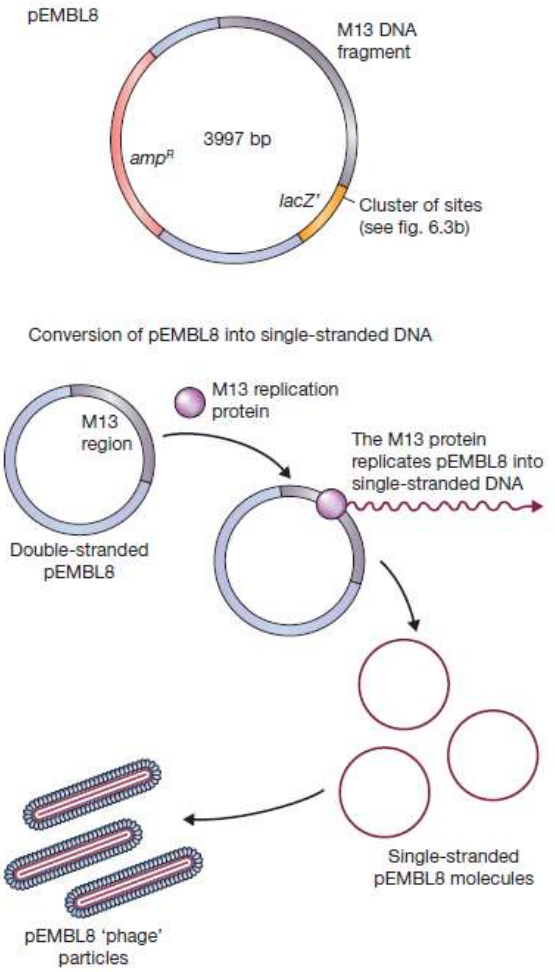
Phagemid vectors are plasmids which have been artificially manipulated so as to contain a small segment of the genome of a filamentous phage, such as M13, fd or f1. The selected phage sequences contain all the cis-acting elements required for DNA replication and assembly into phage particles. They permit successful cloning of inserts several kilobases long (unlike M13 vectors in which such inserts tend to be unstable). They can be replicated in the host cells like plasmids maintaining double stranded replication and high copy number so that large amount of foreign DNA can be recovered. They can also package their single stranded DNA in phage particles because of the presence of M13 origin of replication. Following, transformation or electroporation of a suitable E. coli strain with a recombinant phagemid, the bacterial cells are superinfected with a filamentous helper phage, such as f1, which is required to provide the coat protein. Phage particles secreted from the superinfected cells will be a mixture of helper phage and recombinant phagemids. The mixed single-stranded DNA population can be used directly for DNA sequencing because the primer for initiating DNA strand synthesis is designed to bind specifically to a sequence of the phagemid vector adjacent to the cloning site. A positive selection marker is used to select bacteria containing the phagemid. Fragments of several kilobases of DNA in length can be isolated in single stranded form from the phagemids. They are also utilized for sequencing purposes and for generating templates for site-directed mutagenesis. Commonly used phagemid vectors include the pEMBL series of plasmids and the pBluescript family. λZAP is also an example of phagemid





pEMBL8

pEMBL8 was made by transferring into pUC8 a 1300 bp fragment of the M13 genome. This piece of M13 DNA contains the signal sequence recognized by the enzymes that convert the normal double-stranded M13 molecule into single-stranded DNA before secretion of new phage particles. This signal sequence is still functional even though detached from the rest of the M13 genome, so pEMBL8 molecules are also converted into single-stranded DNA and secreted as defective phage particles. All that is necessary is that the *E. coli* cells used as hosts for a pEMBL8 cloning experiment are subsequently infected with normal M13 to act as a helper phage, providing the necessary replicative enzymes and phage coat proteins. pEMBL8, being derived from pUC8, has the polylinker cloning sites within the *lacZ′* gene, so recombinant plaques can be identified in the standard way on agar containing X-gal. With pEMBL8, single-stranded versions of cloned DNA fragments up to 10 kb in length can be obtained, greatly extending the range of the M13 cloning system.



Viral vectors and plant vectors

- Exploitation of plant viruses as transformation vectors by massive infection may be harmful and even deleterious to the target plants. It is still however able to express and produce foreign proteins.
- Plant viruses must exhibit some of the exemplary features before they are considered as vectors. They should extend their broader host-range, spread of seed transmission and carry additional copies of gene of interest.
- Several viral vectors require suitable modification in order to accommodate extra nucleic acid and also aggressive in infection process. Although several groups of viruses have been identified, some moderate progresses have been made only in two groups. These two groups are Caulimo virus and Gemini virus, which have DNA genome as genetic material.

Cauliflower Mosaic Virus (Caulimovirus):

Cauliflower mosaic virus (CamV) belongs to the group caulimovirus, can be used as potential candidate to deliver foreign gene into the plant. It is perhaps the best studied viruses

among plant virus, which infects several members belonging to Cruciferae family. Cauliflower mosaic virus contains circular double helical DNA as genetic material.

As an infective agent, can cause disease in wide range of commercially important cultivated crops. Cauliflower mosaic DNA has been subjected to a wide range of manipulation. This was the only and first virus to be manipulated and used as a favourable choice for genetic engineering work. Elucidation of 8 kb CamV reveals that, it contains six major and two minor reading frames (Fig. 14.12).

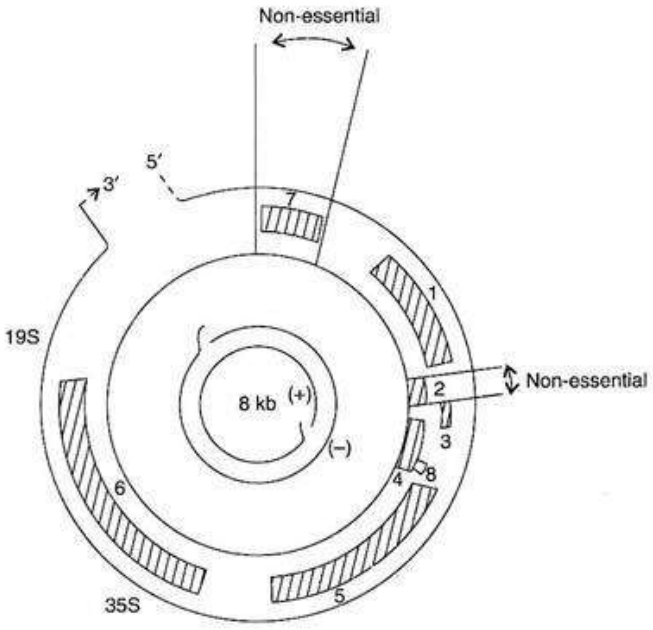


Fig. 14.12. Genetic map of cauliflower mosaic virus.

Presence of ribonucleotide in DNA leads to the conclusion that CamV replication involves the synthesis of negative DNA strand by employing reverse transcriptase and followed by synthesis of positive DNA strand. Once the synthesis of double strand DNA completes, it is then packed into viral particles and continue the cycles of transcription and translation.

CamV Vector:

Cauliflower mosaic virus can be used as a potential vector due to the infective nature of its genetic material. This could be proved by applying viruses on the leaf rubbed with abrasive material. The CamV cannot accommodate foreign DNA, if the size exceeds its normal size. The inserted DNA may destabilize infectious nature of the virus.

Other constraints are the packaging of genome and limitation of the insertion of foreign DNA. Despite the marginal constraints, CamV genome can be packed in nucleosome and is able to undergo transcription by plant RNA polymerase II. The genome of CamV consists of six major and two minor open reading frames (ORF), in tightly packed arrangements.

The two ORF regions, one (ORFII) codes for insect transmission factor and other (PRF VII) with unknown functions can be replaced with gene of interest.

Gemini Virus:

It is a DNA virus, known to infect wide range of economically important and agriculture crops of monocotyledonous and dicotyledonous plants. Several diseases such as maize streak virus and curly top virus are caused by Gemini virus. The genome is single Stranded Circular DNA and its replication takes place by DNA immediately.

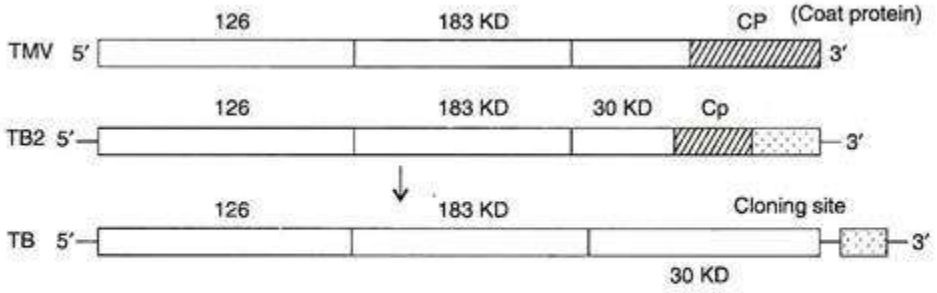
Tobacco Mosaic Virus Based Expression Vector:

Tobacco mosaic virus (TMV) is a RNA virus and shows several advantages by designing expression vector. TMV was the first virus to be purified and sequenced. As far as biohazard is considered TMV could be used as a comparatively safe recombinant virus in the field.

The coat protein of the TMV is one of the most accumulated proteins in plants reachable upto 10% of the dry weight of infected plant. Approximately, under ideal condition, 2000 kg tobacco protein can be produced per acre per year. Moreover, TMV can be purified in crystalline form in substantial quantity by simple methodology.

The single stranded RNA genetic material of TMV encloses 6300 nucleotides with four open reading frames (Fig. 14.13). The filamentous nature of the RNA virus is determined by the length of the viral nucleic acid. Both 183 kD read through protein and 126 kD coat proteins are translated from the 5′ end of the genomic RNA.

These two proteins form replicase complex. In addition to these two proteins, the 30-kD movement protein and 17.5-kD capsid proteins are translated at 3′ region of sub-genomic mRNA during replication.



14.13 Genomic organization of TMV vector (2 and 3)

TMV can be subjected to a wide range of manipulation by replacing the viral coat protein with a foreign protein, for example, replacement of coat protein with reporter gene chloramphenical acetyl transferase (CAT) resulted in a free-RNA virus that generated high CAT activity. Improvisation of TMV vector was achieved by placing CAT gene under the control of a coat protein sub-genomic promoter of TMV into the entire TMV genome.

This recombinant virus produced third sub-genomic mRNA and this was translated into CAT enzyme with high activity. Similarly, another TMV hybrid expression vector TB_2 was designed in which coat protein gene and neomycin phosphotransferase marker gene was placed under the control of sub-genomic promoters of TMV coat protein and ORSV, respectively.

This was referred as extra gene vector. TB_2 effectively produced the foreign protein without any major constraints. Another extra gene in TMV based vector, 4GD-PL, was

developed from tomato green mosaic virus. The 4GD-PL vector was able to express foreign proteins systematically throughout plants.

All these studies demonstrated that proximity of genes to the 3′-untranslated region of the genome increases efficiency of their translation. Another improved TMV vector, 30B, was designed in which the start codon (AUG) of the capsid protein was mutated to AGA, and restriction cloning sites were engineered (40 nucleotide) to provide a full-size sub-genomic RNA promoter.

Possibility of satellite RNA to be used as vector has been considered. They vary in their size between 0.27 and 1.6 kb. They are not indispensable for virus replication. However, their functions can alter pathogenecity of virus.

Cow Pea Mosaic Virus Expression Vector:

Cow pea mosaic virus (CpmV) is also a RNA virus and infects species of legumes. There are two separate positive strand-RNA molecules present in the genetic material of CpmV. The number of nucleotides present in the RNA I and RNA II strand is 5889 and 3480, respectively. Although RNA I alone can replicate on its own but both RNAs are indispensable for infectivity.
The proteins involved in the replication of the virus are encoded by RNA I whereas movement proteins are encoded by RNA II. CpmV capside of both large (L) and small (S) coat protein of 30 copies each are in isohedral symmetry. The two capsid proteins are folded into three antiparallel β-barrel structures.

In the construction of CpmV expression vector, preference was given to the replacement of stable chimeras by insertion of foreign sequences rather than replacement for native residues.
Therefore, in the construction of viable and well refined CpmV vector, precise site of insertion of foreign sequence was given a prime choice by introducing foreign DNA sequence into βB-βC loop of the S protein for most chimeras foreign sequences inserts immediately upstream of proline 23 of the S protein. In view of propagating the chimeras, engineered pCP_2 and pCP_1 are linearised and inoculated on cow pea plants. (Fig. 14.14)

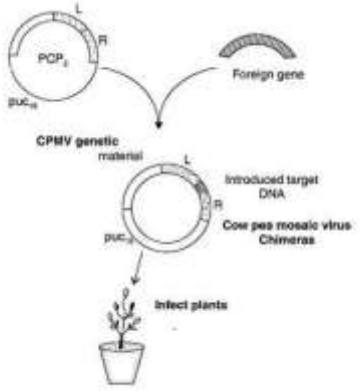


Fig. 14.14 CpmV expression vector construction and infection of plants

POSSIBLE QUESTIONS

8 MARKS

1. What are the basic steps involved in gene cloning? Explain in detail.
2. Discuss and differentiate the roles of cosmids and phagemids.
3. Write in detail about the basic features, classification, copy number of plasmids with examples.
4. Explain in detail about bacteriophage and its life cycle.
5. Give a detailed account on plant viral vectors.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
III-B.Sc., BIOCHEMISTRY
15BCU603A –CORE ELECTIVE II-INTRODUCTION TO BIOTECHNOLOGY
MULTIPLE CHOICE QUESTIONS

UNIT-II

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	The plasmamembrane of protoplasts are dissolved in the presence of	Mg ions	Fe ions	Ca ions	None of the above	**None of the above**
2	The biolistic process currently in widest use employs gunpowder charge to drive	Plastic cylindrical macro projectile	Plastic cylindrical microprojectile	DNA-tungsten suspension	Blank cartridge	**Plastic cylindrical microprojectile**
3	Natural method gene transfer	Microinjection	Macroinjection	Agrobacterium mediated	Biolistic	**Agrobacterium mediated**
4	Artificial method gene transfer	Agrobacterium mediated	Biolistic	Conjugation	Transformation	**Biolistic**
5	Mostly used Artificial method gene transfer	Agrobacterium mediated	Microinjection	Conjugation	Transformation	**Microinjection**
6	Plant gene transfer	Microinjection	Macroinjection	Agrobacterium mediated	Biolistic	**Agrobacterium mediated**
7	The protoplast fusion is done by	PEG method	Electrofusion	Sodium nitrate method	All the above	**All the above**
8	Microinjection in mice was performed at	Male pronuclei	Female pronuclei	Blastocyst	Pronucleus	**Male pronuclei**
9	Microinjection technique prover direct delivery in	Intranuclear region	Intracytoplasmic region	Intro cellular region	Intra species region	**Intranuclear region**
10	Macroinjection technique involved injection of DNA in to	Male pronuclei	Female pronuclei	Wound site with in tissue	Cytoplasm of cell	**Wound site with in tissue**
11	Animal cell gene transfer	Microinjection	Macroinjection	Agrobacterium mediated	Retroviral	**Agrobacterium mediated**
12	Chemical gene transfer method	PEG	DEAE-Dextran	Liposome	All of the above	**All of the above**
13	The microprojectile are commonly DNA coated with	Gold or Tungsten	Silver or Molybdenum	Copper or Zinc	Lithium or Molybdenum	**Gold or Tungsten**
14	The nick translation was performed by	Making ss cuts in ds DNA molecule.	Making ds cuts in ds DNA molecule.	Making ss cuts in ss DNA molecule.	Making no levage in DNA molecule.	**Making ss cuts in ds DNA molecule**.
15	A carrier called a ---------- must be used to deliver the therapeutic gene to the patient's target cells	Fusion agent	Trancription initiater	Vector	Illucitor	**Vector**
16	_____ used to assist the association of the DNA with membrane in Electroporation mediated DNA transfer	Polyethylene glycol	Gun powder	Silicon-Carbide	Calcium	**Polyethylene glycol**
17	Exonuclease III cleaves DNA at	3’	cap site	active site	5’ End	**3’**
18	L aspariginase can be modified by attaching to	Agarose	PEG	Ligand	Ions	**PEG**
19	Biolistic operation cost	35 lakhs	50 lakhs	60 lakhs	10 lakhs	**35 lakhs**
20	The DNA should be mixed with ----------in biolistic process	Calcium phosphate	Biotin	Digitoxigenin	Spermidine	**Spermidine**
21	Homopolymer tailing is a part of	dna sequencing	Gene Cloning	Both	None	**Gene Cloning**
22	Homopolymer tailing used for	cDNA	Probes synthesis	Sequencing	Sequence assembly	**cDNA**
23	Using Homopolymer tailing Joining the DNA is	Artificial method	Sequencing method	Original method	None	**Original method**
24	Homopolymer attached with DNA in_____	Middle	First	End	All of the above	**End**
25	The simplest DNA end of a double strand molecules is called	blunt end	sticky end	both	none of the above	**blunt end**

26	PEG – asparaginase conjugate differ form native enzyme by	it retains 52% catalytic activity	Resistant to proteolytic clevage	Doesnot cause allergy	All the above	**All the above**
27	PEG – asparaginase is used to treat	Tumor	HIV	Artherities	Hydrophilia	**Tumor**
28	A DNA sequence derived from messenger RNA of an expressed gene is called as	tRNA	cDNA	rRNA	none of the options	**cDNA**
29	_____ enzyme is involved in cleaving single base pairs in cDNA.	Bam HI	Eco RI	S1 nuclease	Exonuclease	**S1 nuclease**
30	DNA polymerase used in sequencing should possess	High processivity and high exonuclease activity	High processivity and no exonuclease activity	Low processivity and rapid reaction rate	rapid reaction rate and high exonuclease activity	**High processivity and no exonuclease activity**
31	Longer overhangs are called	cohesive end	sticky end	both	None of the above	**both**
32	Sticky end most often created by	Restriction endonuclease	Polymerase	Plasmids	None of the above	**Restriction endonuclease**
33	The simplest case of an overhang in single nucleotide is called	blunt end	sticky end	both	none of the above	**sticky end**
34	Most of the artificial overhang nucleotide is	G	T	A	C	**A**
35	Electroporation can be otherwords alled as	Electropermeabilization	Induction	Diffusion	Localization	**Electropermeabilization**
36	E**lectropermeabilization**, is a significant increase	electrical conductivity and permeability of the cell plasma membrane	Damage to menbrane	Destroys cell structure	None of the above	**electrical conductivity and permeability of the cell plasma membrane**
37	The proteins that contain a nuclear targeting sequence to direct the T-strand to the nucleus of the transformed cells are	vir D1	vir E2	vir H	vir D2	**vir D2**
38	_____ is the process where electrical impulses of high strength size used for DNA transfer	Microprojectiles	Particle bombardment	Electroporation	Biolistics	**Electroporation**
39	In ---------- method the DNA should be pure	Biolistic	Calcium phosphate	Lipofection	Electrophoraion	**Biolistic**
40	The marker gene present in PBR322 is	Ampr	Kamr	Camr	Metr	**Amp**r
41	Rna can be generated from DNA by a process known as	Reverse transcription	Forward reversion	Mutation	Ligation	**Reverse transcription**
42	Biolistics has been a useful technique for	Testing expression of genes particularly in microbes	Testing expression of genes particularly in earthworm	Testing expression of genes particularly in drosophila	Testing expression of genes particularly in plant	**Testing expression of genes particularly in plant**
43	Type I restriction enzymes cleave DNA at	The point of recognition	1000 to 5000 nucleotides away	25 base pairs away	30 base pairs away	**1000 to 5000 nucleotides away**
44	The co factor needed for type I restriction enzyme were	ATP and Mg 2+	S- Adenosyl Methionine	Both a and b	DNP+	**Both a and b**
45	Restriction enzymes cleave DNA at----------	Specific nucleotide sequence	Interior part of nucleotide sequence	Ends of nucleotide sequence	Both A and B	**Specific nucleotide sequence**
46	Endonuclease are enzymes that cleaves DNA at	Defined sequence	3' end of nucleotide	internal position in random manner	Both a and b	**internal position in random manner**
47	Term endonucleases was coined by----------	Lederberg and Meselson	Lederberg and tatum	Lederberg and yaun	Smith and Nathans	**Lederberg and Meselson**
48	Restriction enzyme from *Escherichia coli* K12 was first isolated from	Meselson and yaun	Lederberg and Meselson	yaun	Meselson	**Meselson and yaun**

49	Recognition site of Alu II	GACGTC	AGCT	TTCGAA	GGATCC	**GACGTC**
50	Recognition site of AluI	AGCT	GAATTC	CCCGGG	TTCGAA	**AGCT**
51	Recognition site of BamHI	GGATCC	CTGCAG	GGCC	TCGA	**GGATCC**
52	Recognition site of EcoRI	GAATTC	AGATCT	TTCGAA	GACGTC	**GAATTC**
53	Recognition site of HindIII	AAGCTT	AGATCT	TTCGAA	GGATCC	**AAGCTT**
54	Recognition site of KpnI	GGTACC	AGATCT	TTCGAA	GGATCC	**GGTACC**
55	Ligases are called	Molecular Scissors	Molecular glue	Molecular tool	None of the above	**Molecular glue**
56	Type I restriction enzyme have	3 subunit	5 subunit	2 subunit	1 subunit	**3 subunit**
57	Type I restriction enzymes are isolated from the organisms	*Escherichia coli* B	*Escherichia coli* K12	Both a and b	*Escherichia coli* dam +	**Both a and b**
58	Type II restriction enzyme cleaves DNA at	Defined recognition site	Random sites	25 bases away	1000 basepairs away	**Defined recognition site**

UNIT-II
SYLLABUS

Introduction of DNA into living cells-methods, microinjection, electroporation, shotgun methods. Transformation and transfection in E.coli – recombinant selection and screening. Maximizing the expression of cloned genes in E.coli- Promoters, Cassettes and production of fusion protein.

Introduction of DNA into living cells-methods

Microinjection

Delivery of nucleic acids to protoplasts or intact cells via microinjection is a labour intensive procedure that requires special capillary needles, pumps, micromanipulators, inverted microscope and other equipment. However, injection into the nucleus or cytoplasm is possible and cells can be cultured individually to produce callus or plants.

In this way selection of transformants by drug resistance or marker genes may be avoided. This method involves skill of the worker to insert needle into the cytoplasm or in the nucleus. The basic technique is similar to that used for animal cell microinjection. In order to microinject protoplasts or other plant cells, the cells need to be immobilized (Fig. 16.3).

The cells are immobilized by:

1. The use of a holding pipette which holds the cells by vacuum.
2. Attachment of cells to poly-L-lysin coated cover slips.
3. Embedding the cells in agarose, agar or sodium alginate.

Glass micropipette are prepared to have openings of about 0.3 µM in diameter and are inserted into plant cell cytoplasm and nuclei with the aid of a micromanipulators device. A syringe like device is used for the controlled delivery of volume (10^{-11} – 10^{-4} 1) into the plant cell.

Most plant cells are injected while keeping inside micro-droplets (2-50 µl) of medium using a chamber which is sterile, vibration free and permits temperature and humidity regulation. A maximum of 100-200 cells per hour can be microinjected by this method.

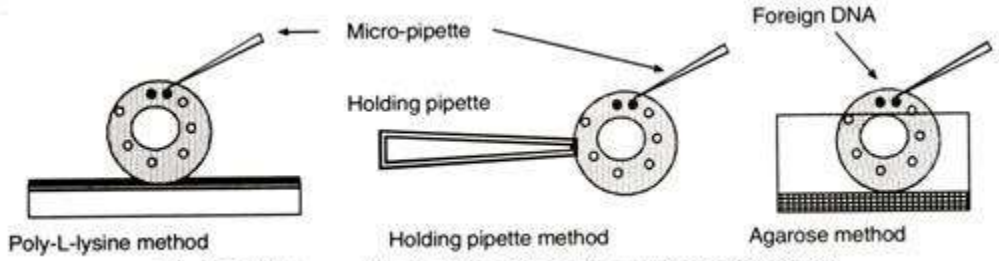


Fig. 16.3. Various methods of immobilizing the cell and microinjection.

The recovery of trans-formants is dependent upon the regeneration ability of the microinjected cells. Different methods have been used to grow injured (microinjected) single cells or protoplasts. Hanging droplets, covered under thin layer of agar or agarose, and micro-

culture have been used (Fig. 16.4). Attempts have been made to inject linear or super-coiled DNA, in cytoplasm or in nucleus. Nuclear injections are found better for transformations.

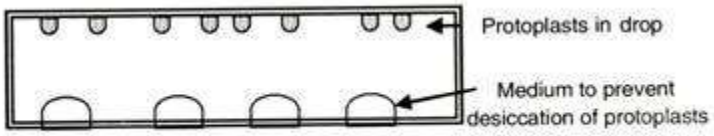


Fig. 16.4. Hanging drop culture method.

Electroporation

Electroporation is a physical transfection method that uses an electrical pulse to create temporary pores in cell membranes through which substances like nucleic acids can pass into cells. It is a highly efficient strategy for the introduction of foreign nucleic acids into many cell types, including bacteria and mammalian cells.

This method is based on the use of the short electrical pulses of high field strength. Electroporation causes the uptake of DNA into protoplasts by temporary permeabilization of the plasma membrane to macromolecules. Protoplasts and foreign DNA are placed in a buffer between two electrodes and a high intensity electric current is passed, the alternating current of about 1 MHz is applied to align the protoplast by di-electrophoresis.

Once aligned, fusion is induced by applying one or more direct current pulses (1-3kV /cm, 10-100 (is), then the alternating field is reapplied briefly to maintain close membrane contact for fusion (Fig. 16.1). Electric field damages membranes and creates pores in membranes. DNA diffuses through these pores immediately after the electric field is applied, until the pores are resealed. Technique is optimized by using appropriate electric field strength (defined as the applied voltage divides by the distance between two electrodes).

The optimum field strength is dependent on the followings:

1. The pulse length of electric current
2. Composition and temperature of the buffer solution
3. Concentration of foreign DNA in the suspension
4. Protoplasts density, and
5. Size of the protoplasts.

It has been demonstrated that the removal of pectin from the plant wall increases the amount of DNA which can be introduced by electroporation. Tobacco mosaic virus was introduced in tobacco protoplasts by this method. Electroporation has been used successfully for transient (when foreign gene which is present in cell but not integrated in the chromosome, shows expression in cytoplasm) and stable transformation (foreign gene integration in host chromosome and is expressed) of protoplasts from a wide range of species.

Plating efficiency (i.e., number of colonies recovered out of number of cells transferred on plates) of electroporated protoplasts grown on selection medium (containing selective marker) can be as high as 0.5%. The highest plant transformation efficiencies have been reported for tobacco, with 0.2% of electroporated leaf mesophyll protoplasts giving rise to transgenic calli. Low transformation efficiency is common in cereals, e.g., in rice 0.002% efficiency was recorded.

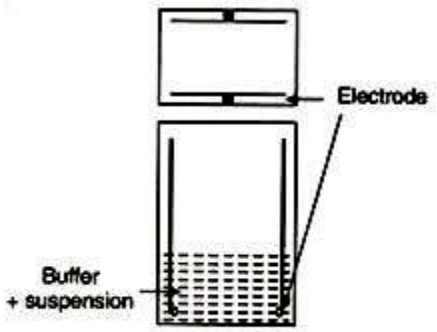


Fig. 16.1. Top (above) and side view (below) of glass cell with electrodes used for electroporation.

Working Protocol

Electroporation is based on a simple process. Host cells and selected molecules are suspended in a conductive solution, and an electrical circuit is closed around the mixture. An electrical pulse at an optimized voltage and only lasting a few microseconds to a millisecond is discharged through the cell suspension. This disturbs the phospholipid bilayer of the membrane and results in the formation of temporary pores. The electric potential across the cell membrane simultaneously rises to allow charged molecules like DNA to be driven across the membrane through the pores in a manner similar to electrophoresis.

The main advantage of electroporation is its applicability for transient and stable transfection of all cell types. Furthermore, because electroporation is easy and rapid, it is able to transfect a large number of cells in a short time once optimum electroporation conditions are determined. The major drawback of electroporation is substantial cell death caused by high voltage pulses and only partially successful membrane repair, requiring the use of greater quantities of cells compared to chemical transfection methods. While more modern instrumentation, such our Neon® Transfection System, overcome high cell mortality by distributing the electrical pulse equally among the cells and maintaining a stable pH throughout the electroporation chamber, optimization of pulse and field strength parameters is still required to balance the electroporation efficiency and cell viability.

Electroporation is based on the principle that high voltage electric pulses can induce cell plasma membranes to fuse. Thus, electroporation is a technique involving electric field-mediated membrane permeabilization. Electric shocks can also induce cellular uptake of exogenous DNA (believed to be via the pores formed by electric pulses) from the suspending solution.

Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).

The basic technique of electroporation for transferring genes into mammalian cells is depicted in Fig. 6.11. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.

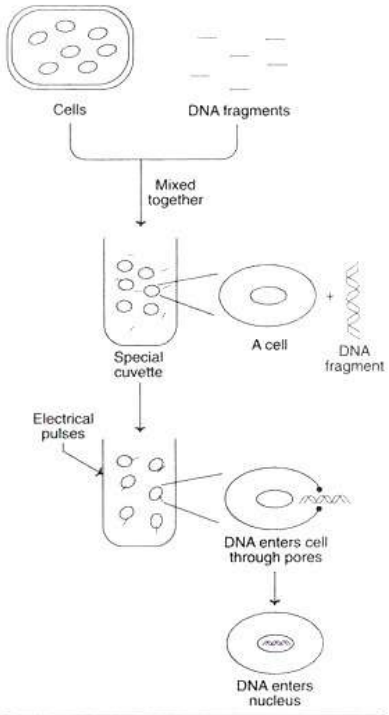


Fig. 6.11 : Gene transfer by electroporation.
(Note : Magnification depicted on right side)

Electroporation is an effective way to transform E.coli cells containing plasmids with insert DNAs longer than 100 kb. The transformation efficiency is around 10^9 transformants per microgram of DNA for small plasmids (about 3kb) and about 10^6 for large plasmids (about 130 kb).

Shot-Gun methods

In recent years, it has been shown that DNA delivery to plant cells is also possible, when heavy metallic pellets (tungsten or gold) coated with the DNA of interest are accelerated to a very high initial velocity (1,400 ft/sec.)

These microprojectiles, normally 1-3 nmm diameter, are carried by a macroprojectile' or the **"bullet"** and accelerated into living plant cells (target cells can be pollen, cultured cells, cells in differentiated tissue and meristems) so that they can penetrate cells walls of intact tissue.

The acceleration is achieved either by an explosive charge (cordite explosion) or by using shock waves initiated by a high-voltage electric discharge. The design of two particle guns used for acceleration of microprojectiles are shown in Fig. 11.17.

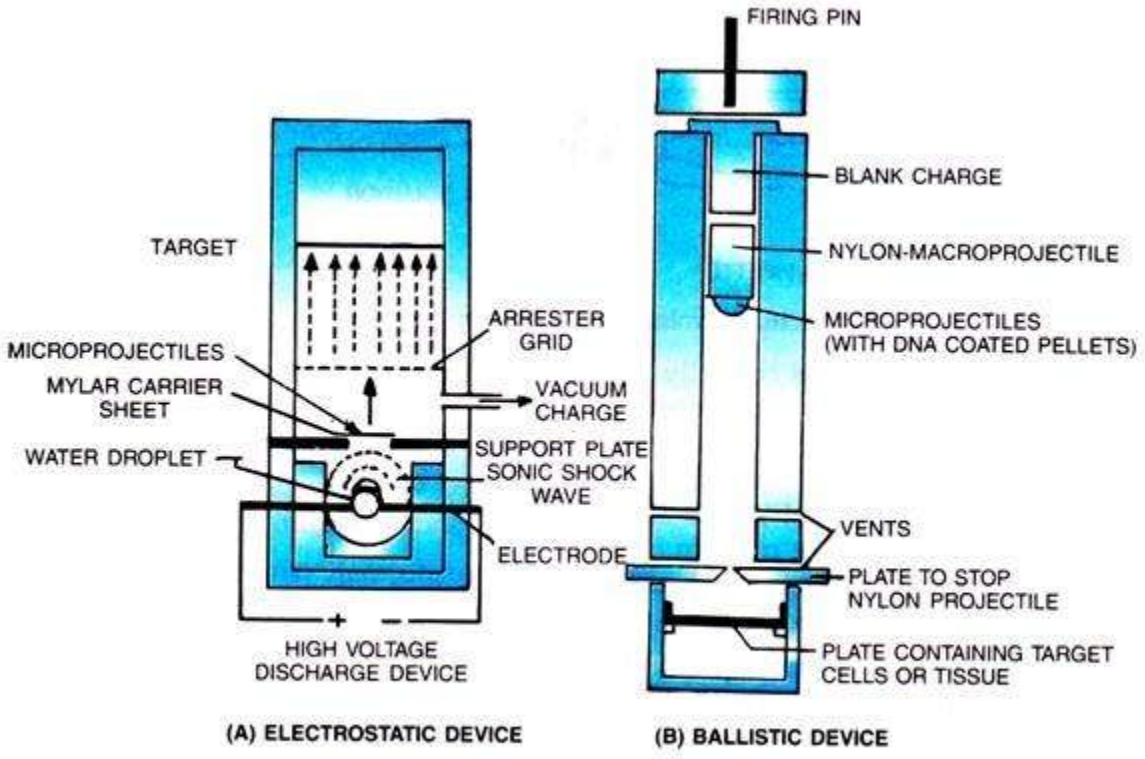


Fig. 11.17. Microprojectile acceleration devices.

The advantages of this method over microinjection include:

(i) Thousands of particles are accelerated at the same time, causing multiple hits resulting transfer of genes into many cells simultaneously.

(ii) Since inact cells can be used, some of the difficulties encounter with the use of protoplasts are automatically circumvented.

(iii) The method is universal in this application, so that cell type, size and shape or the presence/absence of cell walls do not significantly alter its effectiveness. In view of this, particle bombardment method using microprojectiles has a great promise in a variety of plant species, particularly the cereals.

The phenomenon was first discovered in 1928 from Griffiths experiment with Diplococcus pneumoniae. As this historical experiment has been instrumental for identification of DNA as a hereditary material. Briefly, when a suspension containing a mixture of heat-killed virulent, encapsulated cells and live, non-virulent, non-encapsulated cells was injected into a mouse, a small fraction of the live bacteria became transformed into the virulent encapsulated type.

The transforming ability was inherited by the descendants of the newly transformed live strain. It was inferred that when cells of the virulent strain are killed by heat, their chromosomal material which is somehow liberated from heat-killed cells can pass through the cell wall of the living cells and become incorporated in the host chromosome. Although this experiment involved

genes that control presence/absence of the capsule, later on genes controlling other characters could also be transformed by addition of chromosome fragments.

Transformation has proved useful in locating genes in bacteria like Bacillus subtilis, E. coli, Hemophilus influenzae, Rhizobium, Neisseria and others. Attempts have been made to find out if transformation occurs in higher organisms including mammals and man.

All results have been so far negative, except for some special cases where viral chromosome, for example of SV40 (simian virus), which can transform normal human cells in culture into cancerous ones. The mechanism of transformation is not fully known.

Transformation and transfection in E.coli

Process of Transformation

When recipient cells are grown in presence of killed donor cells, transformation is observed. The DNA of donor cells is transferred to recipient cells where it undergoes genetic exchange with recipient chromosomes to produce recombinant progeny.

Analysis of the process indicates that successful transformation depends upon several factors: size of donor DNA fragments which varies in different species of bacteria; molecular configuration of donor DNA which must be double stranded; physiologically competent state of recipient cells which occurs over a limited period in the growth of a culture and the ability to achieve this state is an inherited character; the amount of DNA added per recipient cell, i.e., the frequency of transformation increases with the concentration of DNA up to the point where 10 molecules of DNA per cell are present. Further increase in concentration of DNA seems to have no effect.

Competence of Transformation

When a recipient cell is able to absorb donor DNA and become transformed, it is said to be a competent cell. The development of the competent state appears to be related with cell density. Thus most cells growing in culture become competent when a critical number of cells is attained. Competence therefore, represents a transient phase in the life of a population. Its time of occurrence and duration are characteristic for a bacterial genus.

As competence is acquired by cells in culture, a protein called competence factor is produced which confers competence on other cells. This factor seems to act by changing the cell surface properties either by formation of receptor sites, or increased permeability to donor molecules. Cyclic AMP is also found to play a role in the development of competence. When added to the medium, this compound greatly increases the level of competence among the cells.

Uptake of DNA

The double stranded donor DNA molecules bind to the receptor sites on the recipient cell surface. Both homologous DNA and DNA from an unrelated species will be taken up by Pneumococcus whereas Hemophilus will take up only homologous DNA. The donor fragments are cleaved by endonuclease on the surface of the recipient cell to a size which varies in different bacterial species.

After attachment to the recipient cell wall, the donor DNA is actively transported inside the cell. Soon after uptake, one strand of the donor DNA fragment is degraded so that it becomes single stranded (Fig. 17.1). Immediately there is no transforming activity (eclipse period).

Eventually the fragment pairs with that region of the recipient cell chromosome with which it is homologous. Genetic exchange takes place and a single strand of donor DNA carrying one or more genes from the donor cell becomes integrated in the homologous portion (having corresponding sequence) of recipient DNA. The single stranded segment which breaks of from the recipient DNA is degraded in the cell and lost.

That transformation is a reversible process can be demonstrated experimentally. If donor DNA fragment contains a hypothetical gene t^- and the recipient t^+, the transformed bacteria are found to contain t^-. When these t^- bacteria are used as recipients for donor t^+ DNA, the resulting bacteria become t^+ again.

Transfection

Studies with Bacillus subtilis have shown that when DNA from an animal virus or bacteriophage is used in transformation, intact virus particles are formed inside the recipient bacterial cell. The process is called transfection. In this case there is no need for donor DNA to become integrated into the host chromosome.

When such a bacterium comes in contact with the animal host which the virus is able to infect, it releases the contained virus particles causing infection of the host animal. Experimentally transfection can be assayed by formation of plaques when infected bacteria burst to release the virus progeny.

Linkage and Gene Mapping by Transformation:

Fragments of donor DNA which are involved in transformation can be used for detecting linkage and gene order in bacteria. The method consists of counting the number of double transformants (that is cells transformed for two genes) as well as single transformants produced by a single gene.

Suppose two genes E and F are placed distantly apart on the bacterial chromosome. The probability of both occurring together in the same fragment and producing double transformants is quite low. But a cell can become doubly transformed if it receives two separate donor fragments, one carrying E, the other F.

The probability for such an event would equal the product of their separate probabilities in producing single transformants for E and for F and would be lower than the single events.

But if E and F genes are closely linked, the probability that both are present on the same fragment and produce double transformants is high. When the experiment is performed, the number of single and double transformants will also depend upon the concentration of DNA containing donor fragments that are given to the recipient cells.

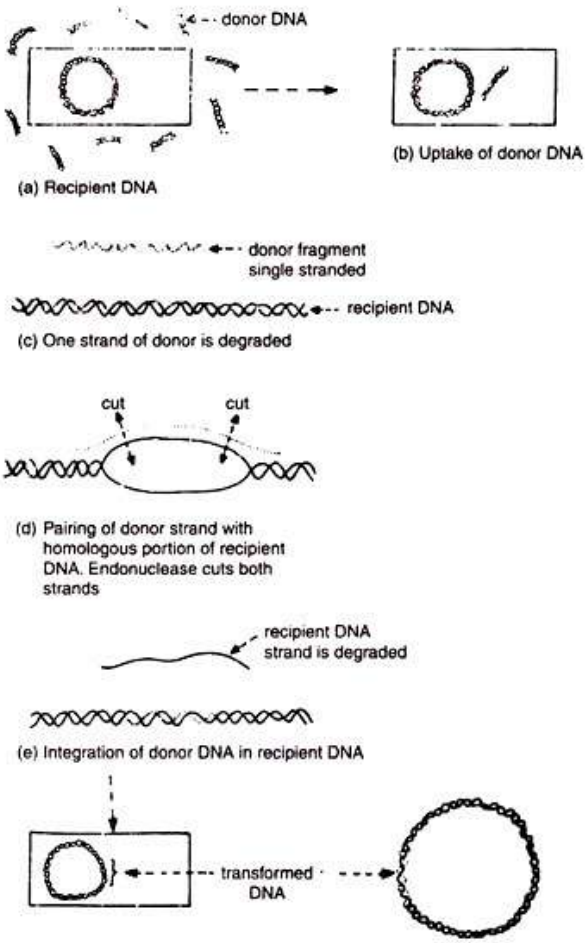


Fig. 17.1 The mechanism of transformation in *E. coli*.

A graph can be plotted to illustrate the curves for single and double transformants with decreasing concentrations of transforming DNA. If genes E and F are linked, the curve for double transformants for E and F must be similar to the curve for single transformants for E and for F.

By using larger fragments of donor DNA it is possible to map gene loci in the vicinity of E and F, and also other genes in the genome. Three gene mapping, similar to the three points cross in higher organisms is also done for mapping genes in bacteria.

Transformation is the method of introducing foreign DNA into bacterial cells (e.g. E.coli). The uptake of plasmid DNA by E.coli is carried out in ice-cold $CaCl_2$ (0-5°C), and a subsequent heat shock (37-45°C for about 90 sec). By this technique, the transformation frequency, which refers to the fraction of cell population that can be transferred, is reasonably good e.g. approximately one cell for 1000 (10^{-3}) cells.

Transformation efficiency

It refers to the number of trans-formants per microgram of added DNA. For E.coli, transformation by plasmid, the transformation efficiency is about 10^7 to 10^8 cells per microgram

of intact plasmid DNA. The bacterial cells that can take up DNA are considered as competent. The competence can be enhanced by altering growth conditions.

The mechanism of the transformation process is not fully understood. It is believed that the $CaCI_2$ affects the cell wall, breaks at localized regions, and is also responsible for binding of DNA to cell surface. A brief heat shock (i.e. the sudden increase in temperature from 5°C to 40°C) stimulates DNA uptake. In general, large-sized DNAs are less efficient in transforming.

Other chemical methods for transformation

Calcium phosphate (in place of $CaCI_2$) is preferred for the transfer of DNA into cultured cells. Sometimes, calcium phosphate may result in precipitate and toxicity to the cells. Some workers use diethyl amino ethyl dextran (DEAE -dextran) for DNA transfer.

Recombinant selection and screening

Mutations are genetic changes or modifications caused by chemical and physical mutagens. Mutations can results from modification of a single base or few bases. However this can result in change or modification of a phenotypic character which can be used to recognize them. This feature is widely used in DNA recombinant technology. Plasmid vectors carry genes for drug resistance, toxin production which can be used to distinguish recombinants. When genes of interest are inserted into the plasmid, the reading frame for the marker genes can be altered. This results in mutants who can be identified using special chemicals/ media.

AMES test is one such method to identify mutants of *Salmonella typhimurium* that cannot produce Histidine. This mutant stain can be cultured only when Histidine is present in the basic medium. This is the standard culture used for testing chemical mutagens. The chemical mutagen is loaded into a well in the centre of a culture plate of inoculated with *Salmonella typhimurium*in a medium lacking Histidine. The chemical diffuses into the medium. A growth indicates that the chemical has induced a mutation in Histidine- stain converting it to Histidine +. Depending on the position of the colony relative to the well containing the chemical, the degree of resistance varies. Colonies growing closer to the well are quite resistant and colonies growing at the periphery indicate that the chemical even at a low concentration can induce **mutation**. This test has its application in pharmaceutical industry to test the effect of drugs; whether it's a mutagen or not.

In **replica plating method** to screen mutants, the organism is subjected to radiation or exposed to chemical to induce the mutation. The mutants can be identified by a reduction in the colony size or change in pigmentation etc. Sub culture is made by replica plating the master culture. The sub cultured plate is subjected to mutation and incubated for growth. Then the plate subjected to mutation is replica plated onto a fresh medium and incubated to observe phenotypic variations. This method can be used to identify the dosage of radiation or chemicals required to cause mutations. If this is repeated with different dosage levels, the finally left colony will be having most resistant cells for the particular mutagen.

Gradient method is used to study the effect of chemical mutagens on bacteria. A medium containing two different concentrations are prepared separately and poured over the same plate

in a slanting position. First the medium with lower concentration of the chemical is poured onto the plate in a slanting position and allowed to solidify. Then the medium with higher concentration of the chemical is poured onto the plate. The plate is inoculated and observed for growth. This is repeated till there is no growth at higher concentration to identify the effective concentration.

Blue white selection is a widely used method in screening recombinants in cloning. This is based on the gene product of lac z gene. The plasmid vectors contain this gene which produces **β galactosidase** enzyme. When a gene is inserted close to **lac z gene**, the reading frame will be distorted and the gene is inactivated. So the transformed cells will not produce this enzyme and are called competent cells. After the recombination, the bacterial cells are grown in a medium containing X gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and **IPTG (Isopropyl β-D-1-thiogalactopyranoside).** IPTG acts as the inducer for lac z gene and enhance the production of β galactosidase. When it is produced, combines with X gal to form a blue colour complex called **5,5'-dibromo-4,4'-dichloro-indigo** which is insoluble. The transformed colonies will appear white in colour and non- transformed cells will appear blue in colour. This method is also called as insertional inactivation of lac z gene.

Hybridization techniques are widely used to identify recombinants. This is based on the ability of nucleic acids hybridize with complementary DNA. The transformed cells are transferred on to a **nitrocellulose membrane** which is subjected to cell lysis. The double stranded DNA is converted to single stranded DNA and immobilized on the membrane. Then it is treated with radiolabelled **probes** complementary to target DNA. If the desired DNA is present, the probes will be hybridized which can be detected by autoradiography.
Apart from these methods, **immunochemical methods** are used to detect protein products to screen recombinants.

Additional Methods for Screening and Selection of Recombinants
Antibiotic resistance
This is one of the simplest selection methods. The plasmid of our interest should contain a specific gene for antibiotic resistance. For example, plasmid pBR322 contains the resistance for ampicillin and tetracycline. If the cells are successfully transformed (if they have taken up the plasmid), they will be resistant to both of these antibiotics, so other unwanted cells which do not contain our gene of interest can be easily eliminated simply by placing the cells on agar medium which contains either one or both of these antibiotics. This method works well also on the mammalian cells.

Insertional inactivation
Insertional inactivation helps us detect the target DNA because it disrupts the coding sequence once it has recombined. For example, if the target DNA gets inserted into the sequence coding for ampicillin resistance (because specific restriction enzyme cuts exactly at that position), it renders the cell which has successfully transformed sensitive to the ampicillin. What we can do next is grow cells in the culturing medium and wait for the colonies to form. We can then make a

replica plate, expose it to the ampicillin and mark which colonies are sensitive, so we can work with them on the original plate.

Plaque morphology

There are some λ vectors (bacteriophage type of vectors) like λgt10 which encode the cI gene. The cI gene codes for the cI repressor proteins which allow phage lambda to transform the bacteria and start the lysogenic cell cycle. This basically means that the phage will live quietly in the cell, integrated into its genome (as a prophage) instead of multiplying itself, destroying the cell and exiting to attack the surrounding cells (lytic cycle). Plaques derived from the cI+ vectors will be turbid because some cells in the lysogenic cycle have survived. However, if the cI gene is inactivated during the process (due to the insertion of our fragment), the plaques formed will be clear and easily distinguishable from the turbid non-recombinants.

Selection of Host Cells for Gene Expression

The nature of a host cell or an organism is as important as the nature of a vector.

The most important requirements of a good host include its suitable cultivation in the laboratory, besides incorporating the vector's genetic material. Several prokaryotes and eukaryotes are employed as hosts to express foreign genes.

Prokaryotic Hosts:

The bacterium Escherichia coli was the first organism to be used in recombinant DNA technology experiments, and continues to be a host of choice for commercial production of proteins. The extensive use of E. coli is mainly due to its high rate of reproduction (the cells double in number, every twenty minutes), besides good knowledge on its biochemistry, physiology and molecular biology.

There are a few disadvantages also in using E. coli as a host. These include a relatively poor export system for proteins and the production of endotoxins which are often difficult to remove (from other useful products). The other host bacterium in use is Bacillus subtilis. This organism is widely employed for commercial production of antibiotics, industrial enzymes, insecticides etc.

Eukaryotic Hosts:

It is often desirable to use eukaryotic organisms, with a well-defined nucleus and cellular organelles, as hosts. The main advantage with eukaryotes is that they bring about several post-translational modifications to make viable and functional proteins. Further, use of eukaryotic hosts is not associated with the generation and interference of toxins which is the case with some prokaryotes. Thus, eukaryotic gene expression systems are preferred for the production of proteins and therapeutic agents that are useful for humans and animals.

The yeast Saccharomyces cerevisiae is widely used as a host for the expression of cloned eukaryotic genes. The other yeasts in use include Kluveromyces lactis, Schizosaccharomyces pombe and Picha pastoris. The insect cells which are infected by baculovirus are in use as hosts in recent years. The baculovirus system can carry and express hundreds of genes in insect cells. Another advantage is that the safety factor since baculoviruses does not infect humans, other vertebrates or plants.

Mammalian cells such as mouse cells can be used as hosts to produce complex proteins with optimal biological functions. But the limitations with mammalian cells are that the techniques are

tedious, often difficult, and also expensive. The manipulation of gene expression, as it is carried out, in prokaryotic and eukaryotic cells.

Maximizing the expression of cloned genes in *E.coli*

Manipulation of Gene Expression in Prokaryotes:

The prime objective of gene cloning is to finally result in the large scale production of proteins for a variety of purposes (industrial, commercial, human health and welfare). This is achieved by the maximal expression of cloned genes through manipulations.

The following are the important features of gene expression that can be considered for manipulation:

i. The presence of regulatable promoters.
ii. The number of copies of cloned genes.
iii. The location of the cloned genes whether inserted into a plasmid or integrated into host genome.
iv. The translation efficiency of the host.
v. The cellular location of the foreign protein and its stability in the host cell.

Some of the strategies that are employed for the manipulation of gene expression in E. coli are discussed hereunder.

Regulatable promoters:

The presence of a strong regulatable promoter sequence is essential for an effective expression of a cloned gene. This is achieved by employing the promoters of E. coli lac (lactose) operon or trp (tryptophan) operon. These promoters have strong affinity for RNA polymerase, and consequently the downstream region (of cloned gene) is transcribed. The promoters thus provide a switch for turning on or turning off the transcription of a cloned gene.

Fusion proteins:

The combination of a foreign protein (encoded by a cloned gene) with the host protein is referred to as a fusion protein. In general, the foreign proteins synthesized are rapidly degraded. This can be reduced by covalently linking a stable host protein to the foreign proteins (i.e., fusion proteins).

The fusion proteins in fact protect the proteolytic degradation of cloned gene product. The synthesis of fusion proteins is achieved by ligating the coding sequences of two genes (cloned gene and host gene). However, it is absolutely essential to ensure that cloned gene contains the correct sequence for the synthesis of the target protein.

Cleavage of fusion proteins:

The fusion proteins, as such, interfere with the biological activity of the target protein. Therefore, these proteins should be cleaved to release the specific desired functional proteins.

Uses of fusion proteins:

The purification of recombinant proteins is much easier in the form of fusion proteins. Fusion proteins are also useful for generating antibodies against target proteins.

Tandem gene arrays:

In general, increase in the number of plasmids (containing cloned gene) proportionately increases the production of recombinant protein. This has a drawback. As the plasmid number increases, the genes coding for antibiotic resistance also increase. The overall effect is that the

regular metabolic activates of the host cell are disturbed for the synthesis of plasmid proteins. Consequently, the yield of cloned gene product is not optimum.

An alternative approach is to clone multiple copies of the target gene on a single plasmid (instead of a single gene on a plasmid). In this manner, tandem arrays of a gene can be created. However, each sequence of the genes should be in correct orientation for transcription and translation.

Efficiency of translation:

The quantity of the cloned gene product produced depends on the efficiency of translation. In general, the binding ability of mRNA with the ribosomal RNA, at translational initiation signal called ribosome binding site determines translation. Thus, the efficiency of translation is better if the binding of mRNA to rRNA is stronger. The actual binding between mRNA and rRNA occurs by complementary base pairing of a sequence of 6-8 nucleotides. To achieve maximum translation, the E. coli expression vectors are designed to possess a strong ribosome binding site.

Stability of proteins:

The half-lives of recombinant proteins are highly variable, ranging from minutes to hours. The stability of proteins can be increased by adding amino acids at the N-terminal end of the proteins. Thus, by attaching methionine, serine and alanine to the N-terminal end, the half-life of β-galactosidase can be increased from 2 minutes to 20 hours! Frequently, a single amino acid addition at N-terminal end stabilizes the protein. The yield of recombinant DNA proteins can be enhanced by increasing half-lives.

Secretion of proteins:

The stability of a protein and its secretion are interrelated. An amino acid sequence (signal peptide) may be attached to a protein to facilitate its secretion through cell membrane. Recombinant proteins secreted into the growth medium can be easily purified.

Integration of cloned DNA into the host chromosome:

The use of plasmids for transcription and translation of cloned DNA imposes a metabolic load on the host. In addition, there is often a chance of losing plasmids during cell multiplication. These problems can be overcome by integrating the cloned DNA directly into host chromosomal DNA. Once the cloned DNA becomes a part of genome, it can be maintained for several generations.

Cloned DNA integration into the host DNA is possible only when there is a complementary sequence of about 50 nucleotides between them. The exchange of DNAs occurs by a recombination process (Fig. 11.1). The cloned DNA lies in the middle of plasmid DNA. On physical contact with chromosomal DNA, base pairing occurs between plasmid DNA (x and y) and chromosomal DNA (x' and y'). And the cloned DNA is transferred to host chromosomal DNA by a physical exchange i.e., recombination.

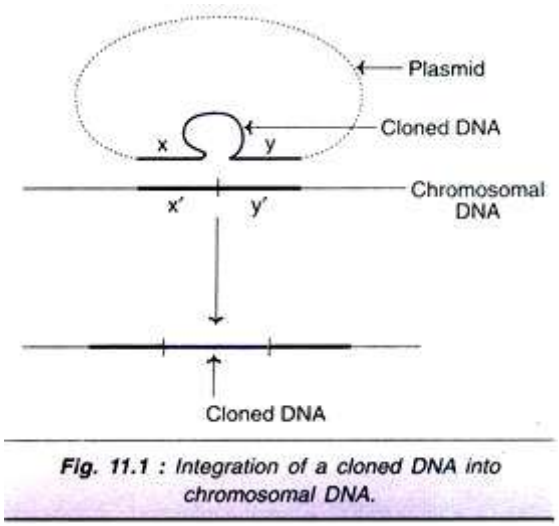


Fig. 11.1 : Integration of a cloned DNA into chromosomal DNA.

Metabolic load:

The presence of cloned DNA alters the metabolism and cellular functions of the host organism. Such metabolic changes are collectively referred to as metabolic load, metabolic drain or metabolic burden. There are several causes for the metabolic load. These include increased utilization of energy for replication and maintenance of plasmids, overproduction of proteins (also drains amino acids, tRNAs), and interference of foreign proteins on the host cell function.

Manipulation of Gene Expression in Eukaryotes:

Expression of cloned genes in eukaryotes has certain advantages. The most important being the ability of eukaryotic organisms to bring about post-translational modifications—glycosylation, phosphorylation, correct disulfide bond formation, proteolytic cleavage etc. Eukaryotic expression systems produce stable and biologically active proteins. This is in contrast to the prokaryotic expression of cloned genes.

In general, the eukaryotic expression of cloned genes is quite comparable to that occurs in the prokaryotes. However, from the technical perspective, it is more difficult to conduct experiments with eukaryotic cells. Many a times, vectors with two distinct origins of replication are used. They serve as shuttle vectors and function in prokaryotic as well as eukaryotic hosts.

The insertion of a foreign DNA into bacterial and yeast cells is referred to as transformation The term transfection is used for the introduction of a foreign DNA into animal cells. The insert DNA in the eukaryotic cells may be associated with vector or integrated into the host chromosomal DNA.

Saccharomyces Cerevisiae— The Yeast in Expressing Cloned Genes:

The common yeast Saccharomyces cerevisiae is widely used as a host for the expression of cloned genes. There are many justifiable reasons for its extensive use.

i. S. cervisiae is single-celled that can be easily grown. Its biochemistry, genetics and physiology are quite known.

ii. It has a naturally occurring plasmid and strong promoters for efficient expression.

iii. S. cerevisiae can bring about many postradiational changes in proteins.

iv. The secreted recombinant proteins can be easily isolated, since very few host proteins are secreted.

v. The U.S. Food and Drug Administration has certified S. cerevisiae as a generally recognized as a safe (CRAS) organism.

As such, S. cerevisiae has been in use for several decades in baking and brewing industries. Biotechnologists work quite comfortably with this yeast to produce a large number of recombinant proteins. These include insulin, a_1 -antitrypsin, hepatitis B virus surface antigen, platelet derived growth factor, fibroblast growth factor and HIV-I antigens. These products are in use as diagnostic agents, vaccines, and therapeutic agents.

Vectors for S. cerevisiae:

There are three types of vectors for S. cerevisiae:

1. Episomal or plasmid vectors.
2. Integrating vectors.
3. Yeast artificial chromosomes (YACs).

1. Plasmid vectors:

Among the vectors, plasmids with single cloned genes are widely used. Manipulation with growth conditions increase the vector stability and expression efficiency. Use of tandem gene arrays has not met with success, since they are unstable.

2. Integrating vectors:

They are basically the integration of cloned genes with chromosomal DNA. These are not frequently used, since the protein production is low.

3. Yeast artificial chromosome (YAC):

Introduced in 1987, YAC is a fragment of yeast DNA that will accept a foreign DNA of about 250-500 kb in length. In fact, the yeast DNA is only about 1% of the total DNA which however, is very important, since it contains three essential genes required for replication. These are the genes for telomere (that protects DNA from nuclease degradation and thus maintains stability), centromere (forms spindles during cell division) and the origin of replication (where DNA polymerase initiates replication). YAC behaves just like a chromosome and replicates.

The construction of the yeast artificial chromosome is depicted in Fig. 11.2. Two opposite ends of a yeast chromosome namely the left telomere and right telomere are chosen. The left telomere is then attached to a centromere. A large segment of the foreign DNA is added and all the three are ligated. Unlike the plasmid vectors, the stability of YAC increases as the size of insert DNA increases.

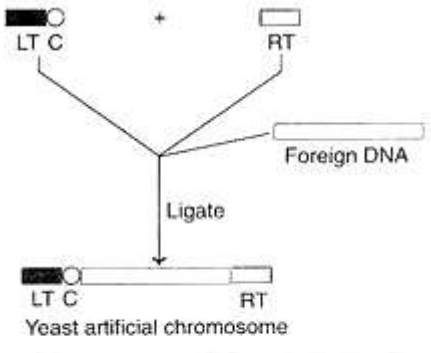


Fig. 11.2 : Construction of yeast artificial chromosome (LT-Left telomere; C-Centromere; RT-Right telomere).

YACs have not been used for commercial production of recombinant proteins. However, they have been employed successfully for physical mapping of genomic DNAs, particularly in human genome project.

Post-translational modifications by S. cerevisiae:

The heterologous proteins synthesized by S. cerevisiae undergo post-translational changes while they are being exported into the extracellular environment. To facilitate protein secretion, a single (leader) peptide is attached to the protein. This peptide is removed by the yeast endoprotease.

Other Yeast Expression Systems:

Despite the very successful use of S. cerevisiae for generating recombinant proteins, there are certain limitations. These include a very low or a limited yield, difficulty in secretion of some proteins and hyper glycosylation. Attempts are being made to explore the utility of other yeasts for the production of hepatitis B virus surface antigen (HBsAg) and bovine lysozyme. The yeast, Hansenula polymorpha, is employed for the synthesis of α- and β-globin chains of human hemoglobin.

Insect Cell Expression Systems:

Cultured insect cells are in use for expressing cloned DNAs. Baculo viruses exclusively infect insect cells. The DNA of these viruses encode for several products and their productivity in cells is very high to the extent of more than 10,000 times compared to mammalian cells. Besides carrying a large number of foreign genes, the baculoviruses can effectively express and process the products formed. Another advantage with these viruses is that they cannot infect humans, other vertebrates or plants. Thus, baculoviruses are safe vectors.

Polyhedrin gene of baculovirus:

The polyhedrin gene is responsible for the synthesis of a matrix protein-polyhedrin. This protein is synthesized in large quantities by baculovirus during the infection cycle. Polyhedrin protects the virus from being inactivated by environmental agents. The promoter for polyhedrin gene is very strong. However, the life-cycle of baculovirus does not depend on the presence of this gene. Polyhedrin gene can be replaced by a cloned gene, and the genetically engineered baculovirus can infect the cultured insect cells.

The cloned gene expresses, and large quantities of recombinant proteins are produced. Because of a close similarity in the post-translational modifications between insects and mammals, biologically active proteins can be produced by this approach. And in fact, by using baculovirus as an expression vector system, a good number of mammalian and viral proteins have been synthesized (Table 11.1).

Table 11.1 Selected examples of recombinant proteins produced by baculovirus expression vector system

Adenosine deaminase
Alkaline phosphatase
Amyloid precursor protein
Anthrax antigen
DNA polymerase α
Erythropoietin
HIV-I envelope protein
Interferons (α, β)
Interleukin-2
Malaria proteins
Pancreatic lipase
Polio virus proteins
Rabies virus proteins
Rhodopsin
Simian rotavirus capsid antigen
Tissue plasminogen activator

Baculovirus expression vector system:

The most commonly used baculovirus is Autograph California multiple nuclear polyhedrosis virus (AcMNPV). It can grow on the insect cell lines (e.g., derived from fall army worm) and produce high levels of polyhedrin or a recombinant protein. The organization of a baculovirus (AcMNPV) transfer vector is shown in Fig. 11.3A. It consists of an E. coli-based plasmid vector along with the DNA of baculovirus. This in turn has AcMNPV DNA, a polyhedrin promoter region, cloning site for insert DNA and polyhedrin termination region.

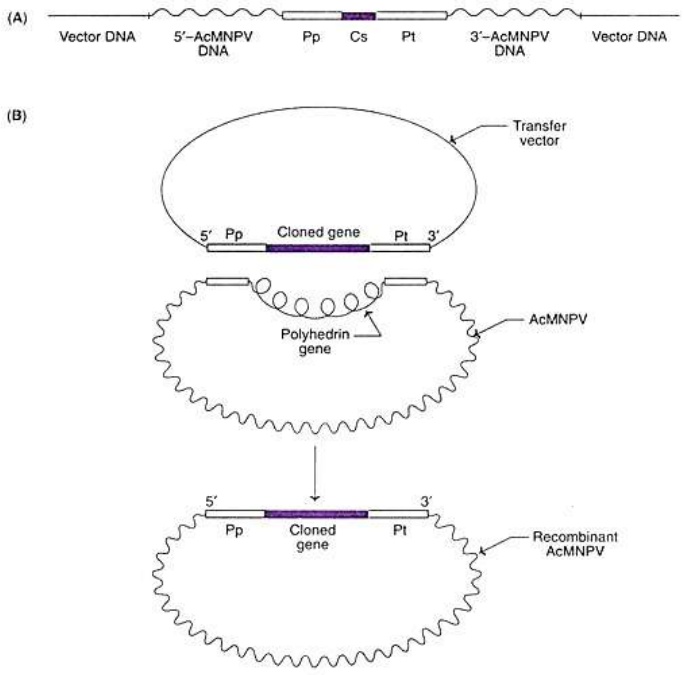


*Fig. 11.3 : Baculovirus expression vector system (A) Organization of baculovirus transfer vector (B) Replacement of the polyhedrin gene of baculovirus with a cloned gene from a transfer vector (AcMNPV–Autographa californica multiple nuclear polyhedrosis virus; Pp–Polyhydrin gene promoter; Cs–Cloning site; Pt–Polyhedrin gene termination; **Note** : The coding region of polyhedrin gene not shown in A).*

When the insect culture cells, transfected with AcMNPV are mixed with transfer vector carrying a cloned gene, a double crossover occurs. The result is that the cloned gene with polyhedrin

promoter and termination sequences gets integrated into AcMNPV DNA (Fig. 11.3B). In this process, polyhedrin gene is lost. The recombinant baculovirus containing cloned gene is isolated. The host insect culture cells, on infection with recombinant baculovirus, produce heterologous proteins. A large number and a wide variety of recombinant proteins (around 500) have been synthesized in the laboratory. A majority of them (>95%) have the requisite post-translational modifications. A selected list of recombinant proteins is given in Table 11.1.

Modifications in the production of recombinant baculovirus:

The original method of creating recombinant baculovirus has undergone several changes. Incorporation of a unique Bsu 361 restriction endonuclease site on the polyhedrin gene increases the yield of recombinant baculovirus production to about 30% from the normal 1%.

Bacmid:

This is shuttle vector for E. coli and insect cell baculovirus. Construction of a recombinant bacmid is a novel approach to carry out all the genetic manipulations including the expression of baculovirus vector in E. coli.

Use of yeast cells:

The genetic manipulations of AcMNPV genome can be done in yeast cells with yeast-insect shuttle vector. Then the recombinant baculovirus is introduced into insect cells.

Mammalian Cell Expression Vectors:

Mammalian expression vectors are useful for the production of specific and authentic recombinant proteins (for use as therapeutic agents). In addition, they are also helpful for studying the function and regulation of mammalian genes. In general, the mammalian expression vectors are quite comparable to other eukaryotic expression vectors. . However, large-scale production of recombinant proteins with engineered mammalian cells is costly.

A diagrammatic representation of mammalian vector is shown in Fig. 11.4. It contains a eukaryotic origin of replication from an animal virus such as Simian virus 40 (SV40) and a prokaryotic origin of replication (from E. coli). The mammalian vector has a multiple cloning site and a selectable marker gene. Both of them are under the control of eukaryotic promoter and polyadenylation sequences.

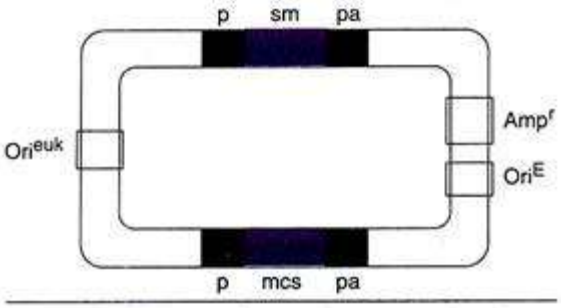


Fig. 11.4 : A diagrammatic representation of mammalian expression vector (p–Promoter sequence; Pa–polyadenylation sequence; mcs–Multiple cloning site; sm-Selectable marker gene; Ori^{euk}–Origin of eukaryotic replication; Ori^{E}–Origin of E. coli replication; Amp^r–Ampicilin resistant marker gene).

These sequences are obtained from either animal viruses (SV40, herpes simplex virus) or mammalian genes (growth hormone, metallothionein). The promoter sequences facilitate the

transcription of cloned genes (at the multiple cloning site) and the selectable marker genes. On the other hand, the polyadenylation sequences terminate the transcription. Ampicillin resistant marker gene can be used for selecting the transformed E. coli cells.

Markers for mammalian expression vectors:

There are several markers in use for the selection of transformed mammalian cells. The bacterial gene (Neo^r) that encodes for neomycin phosphotransferase is frequently used. The other markers are the genes that encode for the enzyme dihydrofolate reductase (DHFR), and glutamine synthetase (GS).

POSSIBLE QUESTIONS

8 MARKS

1. Explain the methods of microinjection and electroporation in detail.
2. Explain in detail about maximizing the expression of cloned genes in *E.coli.*
3. Explain the methods for to introduce DNA into the living cells in detail.
4. Write in detail about transformation and transfection in *E.coli*
5. Explain the method of shotgun in detail.
6. Write a detailed note on production of fusion protein.
7. What are the methods employed to introduce DNA into the living cells? Explain the methods in detail with respective diagram.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
III-B.Sc., BIOCHEMISTRY
15BCU603A –CORE ELECTIVE II-INTRODUCTION TO BIOTECHNOLOGY
MULTIPLE CHOICE QUESTIONS

UNIT-III

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	Most of the artificial overhang nucleotide is	G	T	A	C	**A**
2	Longer overhangs are called	cohesive end	sticky end	both	None of the above	**both**
3	Sticky end most often created by	Restriction endonuclease	Polymerase	Plasmids	None of the above	**Restriction endonuclease**
4	The probes can be Non raollabelled by	Toluene	Biotin	Epinephrin	Globulins	**Biotin**
5	Northern hybridization is used to measure	The amount of RNA	Size of protein	Amount of DNA	Amount of microsatelites	**The amount of RNA**
6	Fragmentation, ligation, transfection, and screening/selection are the steps involved in	Mapping	Cloning	Sequencing	Sequence assembly	**Cloning**
7	Homopolymer tailing is a part of	dna sequencing	Gene Cloning	Both	None	**Gene Cloning**
8	Homopolymer tailing used for	cDNA	Probes synthesis	Sequencing	Sequence assembly	**cDNA**
9	The simplest DNA end of a double strand molecules is called	blunt end	sticky end	both	none of the above	**blunt end**
10	The simplest case of an overhang in single nucleotide is called	blunt end	sticky end	both	none of the above	**sticky end**
11	While sequencing, DNA is denatured and the resulting fragments separated (with a resolution of just one nucleotide from longest to shortest by	Chromatography	gel electrophoresis	lipofection	electroporation	**gel electrophoresis**
12	DNA bands can be detected by exposure to _____light and the DNA sequence can be directly read off the gel.	X-rays or UV	normal light	Radiation	Fluorescent	**X-rays or UV**
13	The oligonucleotide primer is extended using a _____enzyme that replicates DNA	DNA ligase	DNA polymerase	helicase	RNA polymerase	**DNA polymerase**
14	In sequencing the fragments are size-separated by _____, or more commonly now, in a narrow glass tube (capillary) filled with a viscous polymer.	Electrophoresis	electroporation	lipofection	none	**Electrophoresis**
15	Which blotting technique is used for protein?	Northern blotting	Southern blotting	Western blotting	Eastern blotting	Northern blotting
16	The first description of northern blotting was published by	EM Southern	Alwine et al	Karry mullois	Hogness	**Alwine et al**
17	The most effective denaturating agent of RNA is	Formaldehyde	Dimethyl sulphide	Glyonol/ Formamide	Guanidine thiocyanide	**Glyonol/ Formamide**
18	The commonly used membrane in northern blotting are	Polyehylene and nylon	Nylon and nitreocellulose	Terylene and nitrocellulose	Nylon and terylene	**Nylon and nitreocellulose**
19	The original name of nylon is	Nylon	Polystyrene	Artificial	Filter 66	**Polystyrene**
20	Which of the following is tracking dye	Methylene blue	Malachite green	Broophenol blue	Xylene cyanol	**Methylene blue**
21	Nonsense suppression is a speciao case of complementation operating only in the instance of	Frame Shift mutation	Transtition mutation	Transversion	Nonsense mutation	**Nonsense mutation**

22	Pores are formed when the voltage a	exceeds its diele	Exceds its trielectric value	Falls below the dielecric value	Maintains at the dielectric value	exceeds its dielectric strength.
23	In molecular biology, the process of electroporation is often used for the	transformation of bacteria,	yeast,	plant protoplasts	All the above	**All the above**
24	The marker gene present in PBR322 is	Ampr	Kamr	Camr	Metr	**Amp**r
25	The southern hybridization results was read by	UV transilluminate r	Autoradiograp hy	Stainiing	Visible light	**Autoradiograp hy**
26	The polysaccharide contained in agarose is	Sucrose	Galactose	Fructose	Maltose	**Galactose**
27	The glyosidic linkage in Agarose is	(1,2) β (1 , 4)	(1,2) β (1 ,3)	(1,3) β (1 , 4)	(1,4) β (1 , 6)	**(1,4) β (1 , 6)**
28	The mostly preferred buffer in AGE	TAE	TBE	TPE	TE	**TAE**
29	The DNA should move from	Negative to positive	Positive to negative]Positi ve to neutral	Negative to neutral	Positive to neutral	**Negative to positive**
30	Selection by complementation is the existence of	Auxotroph	Prototroph	Heterotroph	Lethal genes	**Auxotroph**
31	The crucial step in northern blotting is	Transfer of denatured RNA from gel to membrane	Denaturation of mRNA	Seperation through AGE	Isolation of m RNA	**Isolation of m RNA**
32	Southern blotting technique was identified by	Southern	Kary Mullis	Lederberg	Guray	**Southern**
33	DNA blotting technique is used to study about	RNA	Proteins	DNA	Lipids	**DNA**
34	The upward and backward transfer of DNA into membrane in southern blotting was performed by	Upward capillary transefer	Downward capillary trtansfer	Bidirection transfer	Vaccum transfer	**Bidirection transfer**
35	The method used when the target DNA fragment when present in higher concentration is	Capillary method	Vaccum blotting	Bidirectional blotting	Transfer in Alkaline buffer	**Bidirectional blotting**
36	Site directed mutagenesis inverted	1978	1988	1968	1998	**1978**
37	The type of mutagenesis involves the cleavage by a restriction enzyme	Oligo nucleotide mutagenesis	Cassette Mutagenesis	Directed mutagenesis	None	**Cassette Mutagenesis**
38	_____ membrane for western blotting	Nitrocellulose	Golden	Tungsten	Filter paper	**Nitrocellulose**
39	Western blotting used for which technique	Blood grouping	ELISA	Monoclonal antibody	None of the above	**ELISA**
40	Molecular weight of a biological compound can be determined by	Cellulose acetate electrophoresi s	Immuno electrophoresis	Starch gel electrophoresi s	SDS poly acryl amide gel electrophores is	**SDS poly acryl amide gel electrophoresis**
41	---------- is the sample used for nuclec acid blotting	DNA	RNA	Protein	Both DNA and RNA	**Both DNA and RNA**
42	For nucleic acid hybridization nucleic acid is digested with---------- restriction enzyme	One	Two	More than two	Other than restriction enzymes	**More than two**
43	---------- refers to blotting of electrophoresed protein bands fron SDS PAGE to membrane	Southern blotting	Northern blotting	Western blotting	Dot blotting	**Western blotting**
44	In which technique purified nucleic acid sample directly applied to nitrocellulose filter	Southern blotting	Northern blotting	Western blotting	Dot blotting	**Dot blotting**
45	Site directed mutagenesis used for	Genetic engineering	Protein engineering	Pharmaceutic al industries	All the above	**All the above**

46	SDS	is an anionic detergent	binding imparts large negative charge	disrupts macromolecules having hydrophobic interactions	All the above	**All the above**
47	In electrophoresis,β mercaptoethanol is added to disrupt	Hydrophobic interactions	Hydrogen bonds	Disulphide linkages	Vanderwaals forces.	**Disulphide linkages**
48	The process of transferring DNA from gel to nitro cellulose paper is called	Northern blotting	Southern blotting	Eastern blotting	Western blotting	**Southern blotting**
49	The technique developed by E.M. Southern used to identify any fragment containing a given gene sequence	Northern blotting	Southern blotting	Western blotting	PCR	**Southern blotting**
50	The technique used for amplification of specific DNA sequences	Southern blotting	Recombinant gene technology	Polymerase chain reaction	Western blotting	**Polymerase chain reaction**
51	The PCR method in site directed mutagenesis involves the use of	Forward primers	Reverse primers	Oligonucleotide primers	Resolvase	**Oligonucleotide primers**
52	Oligonucleotides are often used as probes for detecting	complementary DNA or RNA	For the detection of genes	For the detection of restriction sites	For detecting the degree of hybridization	**complementary DNA or RNA**
53	The process of determining the nucleotide order of a given DNA fragment is called as_____	DNA sequence	Genome assembly	DNA amplification	Chain termination	**DNA sequence**
54	For over thirty years a huge fraction of DNA sequencing has been achieved using	Shotgun sequencing	chain termination	gilbert method	chemical degradation	**chain termination**
55	The classical chain termination method or _____ first involves preparing the DNA to be sequenced as a single strand	Gilbert method	PCR method	Sangers method	None	**Sangers method**
56	Taq DNA polymerase is	Thermostable DNA polymerase	Used in PCR technique	A ligase	All the above	**Thermostable DNA polymerase**
57	Nucleic acid hybridization techniques can be applied to	DNA – DNA	DNA – RNA	RNA – RNA	All of the above	**All of the above**
58	Taq polymerase is obtained from	Thermus thermophiles	Thermus aquaticus	Bacillus thuringenis	None	**Thermus aquaticus**
59	Western blotting was developed by	Smithies	Alwine	Towbin	Southern	**Towbin**
60	Non-Radioactive labelling probes can be of	Biotin	Digoxigenin	Fluorescent molecules	All the above.	**All the above.**

UNIT-III
SYLLABUS

DNA sequencing-Sanger and Maxim Gilbert method. PCR- techniques and applications. DNA foot and finger printing- applications. Hybridization probes- radiolabelled and non-radiolabelled.Hybridization techniques- Southern, Northern, Western blotting techniques.Site directed mutagenesis. DNA microarray.

DNA sequencing

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. Before the development of direct DNA sequencing methods, DNA sequencing was difficult and indirect. The DNA had to be converted to RNA, and limited RNA sequencing could be done by the existing cumbersome methods. Thus, only shorter DNA sequences could be determined by this method. Using this method, Walter Gilbert and Alan Maxam at Havard University determined that the Lac operator is a 27 bp long sequence.

The development of direct DNA sequencing techniques changed the scope of biological research. The evolution of DNA sequencing technology from plus-minus sequencing to pyro-sequencing within about 20 years parallels the progress in biology from molecular biology to genomics.

The development of DNA sequencing techniques with enhanced speed, sensitivity and throughput are of utmost importance for the study of biological systems. Sequence determination is most commonly performed using di-deoxy chain termination technology. Pyro-sequencing, a non-electrophoretic real- time bio-luminometric method for DNA sequencing has emerged as a state of the art sequencing technology.

This technology has the advantage of accuracy, ease of use, and high flexibility for different applications. Pyro-sequencing allows the analysis of genetic variations including SNPs, insertion/deletions and short repeats, as well as assessing RNA allelic imbalance, DNA methylation status and gene copy number.

1. Sanger's Method:

The first DNA sequencing method devised by Sanger and Coulson in 1975 was called plus and minus sequencing that utilized E. coli DNA pol I and DNA polymerase from bacteriophage T4 with different limiting triphosphates. This technique had a low efficiency. Sanger and co-worker (1977) eventually invented a new method for DNA sequencing via enzymatic polymerization that basically revolutionized DNA sequencing technology.

The most popular method for doing this is called the dideoxy method or Sanger method (named after its inventor, Frederick Sanger, who was awarded the 1980 Nobel prize in chemistry [his second] for this achievement). Finding a single gene amid the vast stretches of DNA that make up the human genome – three billion base-pairs' worth – requires a set of powerful tools. These tools include genetic maps, physical maps and DNA sequence which is a detailed description of the order of the chemical building blocks, or bases, in a given stretch of DNA.

Scientists need to know the sequence of bases because it tells them the kind of genetic information that is carried in a particular segment of DNA. For example, they can use sequence information to determine which stretches of DNA contain genes, as well as to analyze those genes for changes in sequence, called mutations, that may cause disease.

The first methods for sequencing DNA were developed in the mid-1970s. At that time, scientists could sequence only a few base pairs per year, not nearly enough to sequence a single gene, much less the entire human genome. By the time the HGP began in 1990, only a few laboratories had managed to sequence a mere 100,000 bases, and the cost of sequencing remained very high. Since then, technological improvements and automation have increased speed and lowered cost to the point where individual genes can be sequenced routinely, and some labs can sequence well over 100 million bases per year.

DNA is synthesized from four deoxynucleotide triphosphates. The top formula shows one of them: deoxythymidine triphosphate (dTTP) (Fig. 23.7). Each new nucleotide is added to the 3′ – OH group of the last nucleotide added.

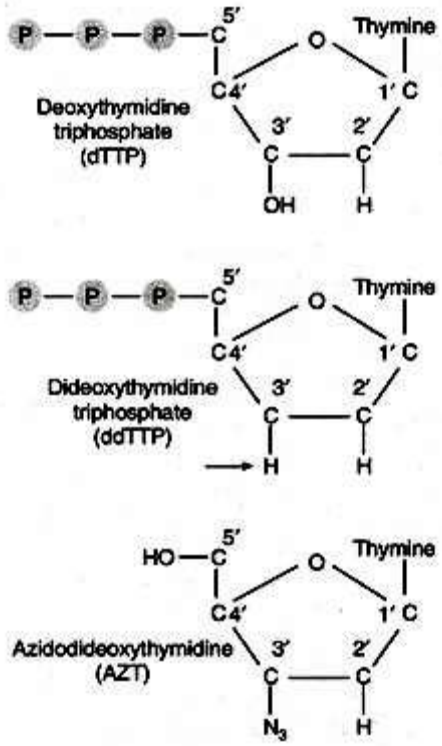


Fig. 23.7. Structure of dideoxynucleotides.

The dideoxy method gets its name from the critical role played by synthetic nucleotides that lack the -OH at the 3′ carbon atom. A dideoxynucleotide (dideoxythymidine triphosphate – ddTTP as shown here) can be added to the growing DNA strand. When it is added it stops chain elongation because there is no 3′ -OH for the next nucleotide to be attached. For this reason, the dideoxy method is also called the chain termination method.

The bottom formula shows the structure of azidothymidine (AZT), a drug used to treat AIDS. AZT (which is also called zidovudine) is taken up by cells where it is converted into the

triphosphate. The reverse transcriptase of the human immunodeficiency virus (HIV) prefers AZT triphosphate to the normal nucleotide (dTTP). Because AZT has no 3′ -OH group, DNA synthesis by reverse transcriptase halts when AZT triphosphate is incorporated in the growing DNA strand. Fortunately, the DNA polymerases of the host cell prefer dTTP, so side effects from the drug are not as severe as might have been predicted.

The Procedure:

The DNA to be sequenced is prepared as a single strand (Fig. 23.8).

This template DNA is mixed with the following:

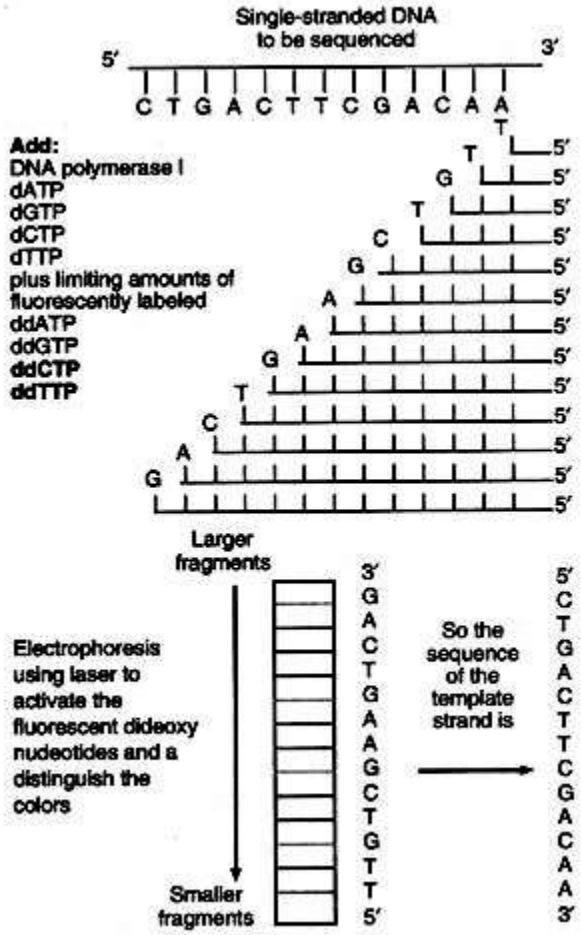


Fig. 23.8. Sanger's method of DNA sequencing.

(a) A mixture of all four normal (deoxy) nucleotides in sample quantities

i. dATP

ii. dGTP

iii. dCTP

iv. dTTP

(b) A mixture of all four dideoxynucleotides, each present in limiting quantities and each labeled with a "tag" that fluoresces a different colour:

i. ddATP

ii. ddGTP
iii. ddCTP
iv. ddTTP

(c) DNA polymerase I:

Because all four normal nucleotides are present, chain elongation proceeds normally until, by chance, DNA polymerase inserts a dideoxy nucleotide instead of the normal deoxynucleotide. If the ratio of normal nucleotide to the dideoxy versions is high enough, some DNA strands will succeed in adding several hundred nucleotides before insertion of the dideoxy version halts the process.

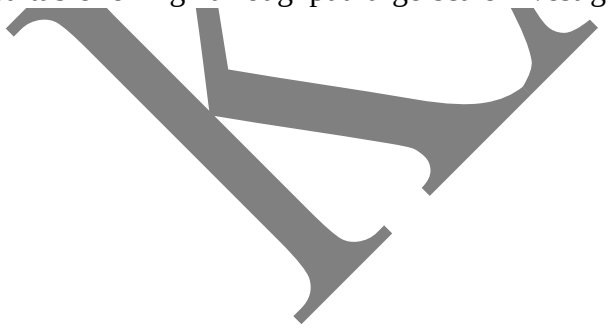
At the end of the incubation period, the fragments are separated by length from longest to shortest. The resolution is so good that a difference of one nucleotide is enough to separate that strand from the next shorter and next longer strand. Each of the four dideoxynucleotides fluoresces a different colour when illuminated by a laser beam and an automatic scanner provides a printout of the sequence.

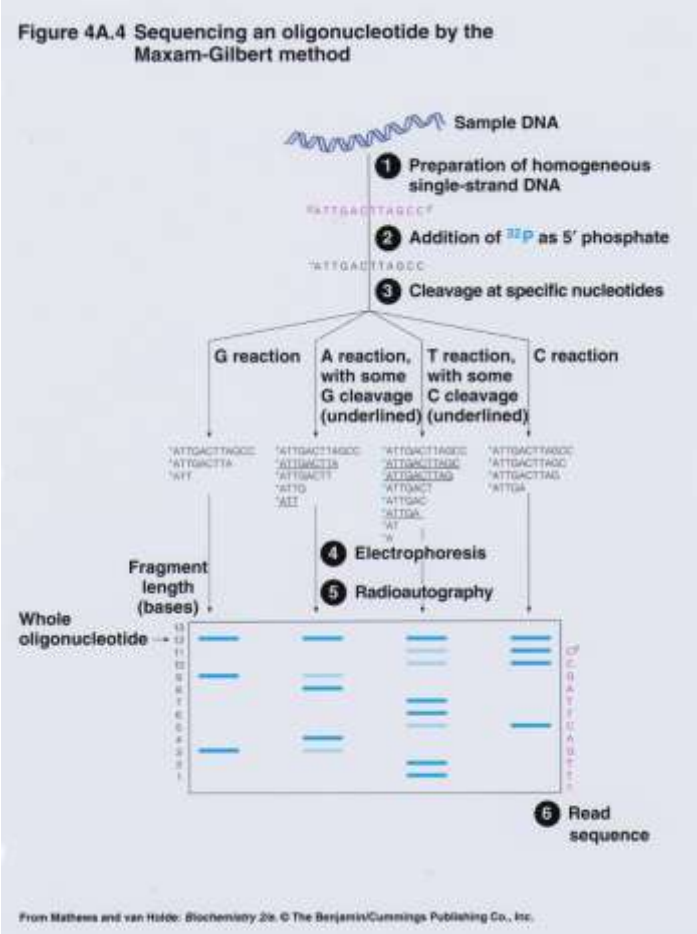
2. Maxam and Gilbert Method:

In 1977, Maxam and Gilbert described a sequencing method based on chemical degradation at specific locations of the DNA molecule. The end labeled DNA fragments are subjected to random cleavage at adenine, cytosine, guanine or thymine positions using specific chemical agents and the products of these fours reactions are separated using polyacrylamide gel electrophoresis (PAGE). As in Sanger method, the sequence can be easily read from four parallel lanes in the sequencing gel.

Double stranded or single stranded DNA from chromosomal DNA can be used as template. Originally, end labeling was done with P phosphate or with a nucleotide linked to P and enzymatically incorporated into the end fragment. The read length is up to 500bp. The chemical reactions in the technique are slow and involved hazardous chemicals that require special handling in the DNA cleavage reaction.

As in Sanger's method, additional cautions in Maxam and Gilbert method include purification and separation of DNA fragments and higher analysis time. Therefore, this technology is not suitable for high throughput large-scale investigation.





3. Hybridization Method:

Ed Southern's (1990) sequencing by hybridization technique relies on detection of specific DNA sequences using hybridization of complementary probes. It utilizes a large number of short nested oligonucleotides immobilized on a solid support to which the labeled sequencing template is hybridized. The target sequence is deduced by computer analysis of the hybridization pattern of the sample DNA.

DNA sequence can also be analyzed by sequencing by synthesis. Sequencing by hybridization makes use of a universal DNA microarray, which harbors all nucleotides of length k (called "k-words", or simply words when k is clear). These oligonucleotides are hybridize to an unknown DNA fragment, whose sequence one would like to determine.

Under ideal conditions, this target molecule will hybridize to all words whose Watson-Crick complements occur somewhere along its sequence. Thus, in principle, one would determine in a single microarray reaction the set of all k-long substrings of the target and try to infer the sequence from those data.

The average length of a uniquely resconstructible sequence using an 8-mer array is <200 bases, far below a single read length on commercial gel-lane machine. The main weakness of sequencing by hybridization is ambiguous solutions-when several sequences have the same spectrum; there is no way to determine the true sequence.

4. Pal Nyren's Method:

In 1996, Pal Nyren's group reported that natural nucleotide can be used to obtain efficient incorporation during a sequencing-by-synthesis protocol. The detection was based on the pyrophosphate (inorganic biphosphate) released during the DNA polymerase reaction, the quantitative conversion of pyrophosphate to ATP by sulfurylase and the subsequent production of visible light by firefly luciferase.

The first major improvement was inclusion of dATPaS in place of dATP in the polymerization reaction, which enabled the pyrosequencing reaction to be performed in homogeneous phase in real time.

The non-specific signals were attributed to the fact that dATP is a substrate for luciferase. Conversely, dATPaS was found to be inert for luciferase, yet could be incorporated efficiently by all DNA polymerases tested. The second improvement was the introduction apyrase to the reaction to make a four-enzyme system. Apyrase allows nucleotides to be added sequentially without any intermediate washing step.

Pyrosequencing nonelectrophoretic real-time DNA sequencing method is based on sequencing by synthesis based on the pyrophosphate (inorganic biphosphate) released during the DNA polymerase reaction.

In a cascade of enzymatic reaction, visible light is generated that is proportional to the number of incorporated nucleotides. The cascade starts with a nucleic acid polymerization reaction in which inorganic bip-hosphate (PPi) is released as a result of nucleotide incorporation by polymerase.

The released PPi is subsequently converted to ATP by ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin and generate light. The light so generated is captured by a CCD camera and recorded in the form of peaks known as pyrogram (compared with electropherograms in Sanger's method). Because the added nucleotide is known the sequence of template can be determined.

Standard pyrosequencing uses the Klenow fragment of E. coli DNA pol I, which is relatively slow polymerase. The ATP sulfurylase used in pyrosequencing is a recombinant version from the yeast and the luciferase is from the American firefly. The overall reaction from polymerization to light detection takes place within three to four seconds at real time.

One pmol of DNA in a pyrosequencing reaction yields 6×10^{11} ATP molecules which in turn, generate more than 6×10^{9} photons at a wavelength of 560 nm. This amount of light is easily detected by a photodiode, photomultiplier tube or a CCD camera. Pyrosequencing technology has been further improved into array-based massively parallel microfluidic sequencing platform.

5. Automatic DNA Sequencer:

A variant of the above dideoxy-method was developed, which allowed the production of automatic sequencers. In this new approach, different fluorescent dyes are tagged either to the oligonucleotide primer (dye primers) in each of the four reaction tubes (blue for A, red for C, etc), or to each of the four ddNTPs (dye terminators) used in a single reaction tube: when four tubes are used, they are pooled.

After the PCR reaction is over, the reaction mixture is subjected to separation of synthesized fragments through electrophoresis (Fig. 23.9). Depending upon the electrophoretic system used, whether slab gel electrophoresis or capillary electrophoresis, following two types of automatic sequencing systems have been designed.

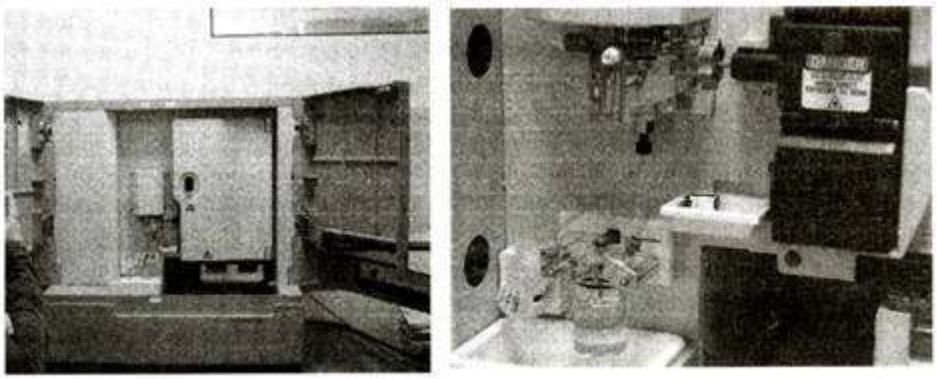


Fig. 23.9. Automated DNA sequencer (left) and details of sample loop (right).

6. Slab Gel Sequencing Systems:

These systems make use of ultrathin (75 µm) slab gels and involve running of atleast 96 lanes per gel. In these systems, automation in sample loading of sequencing gels has also been achieved, by using a plexiglass block having wells that are same distance apart as the comb teeth cut in a porous membrane that is used as a comb for drawing samples by capillary action.

Each well in plexiglass block is filed with a sample (PCR dideoxy-reaction mixture), so that when the porous membrane comb is lowered onto the sample wells in the pexiglass the samples are drawn up automatically into the comb teeth by capillary action.

Using this approach of employing porous combs, automated loading of up to 192, 384 or 480 samples per gel has been achieved. The porous comb with the samples is placed between the glass plates of the gel apparatus above the flat surface of the polymerized gel and the samples are driven from the comb into the gel by electrophoresis.

7. Capillary Gel Electrophoresis:

In these systems, slab gel electrophoresis is replaced by capillary gel electrophoresis to analyse DNA samples. In these systems, instead of scanning DNA as it migrates through 96 lanes each in a series of 96 capillary tubes, DNA fragments pass are scanned.

In the original models of the above old slab gel machines, gels must be poured and reagents frequently reloaded, interrupting the sequencing.

In capillary gel sequencing systems, on the other hand, the robot moves the DNA samples and reagents through the tubes continuously, requiring attention only once a day. The system produces a steady flow of data, each signal representing one of the four DNA bases (adenine, cytosine, guanine and thymine).

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a laboratory (in vitro) technique for generating large quantities of a specified DNA.

Obviously, PCR is a cell-free amplification technique for synthesizing multiple identical copies (billions) of any DMA of interest. Developed in 1984 by Karry Mullis PCR is now considered as a basic tool for the molecular biologist. As is a photocopier a basic requirement in an office, so is the PCR machine in a molecular biology laboratory!

Principle of PCR:

The double-stranded DNA of interest is denatured to separate into two individual strands. Each strand is then allowed to hybridize with a primer (renaturation). The primer-template duplex is used for DNA synthesis (the enzyme- DNA polymerase). These three steps— denaturation, renaturation and synthesis are repeated again and again to generate multiple forms of target DNA.

Technique of PCR:

The essential requirements for PCR are listed below:

1. A target DNA (100-35,000 bp in length).
2. Two primers (synthetic oligonucleotides of 17-30 nucleotides length) that are complementary to regions flanking the target DNA.
3. Four deoxyribonucleotides (dATP, dCTP, dCTP, dTTP).
4. A DNA polymerase that can withstand at a temperature upto 95° C (i.e., thermo-stable).

The reaction mixture contains the target DNA, two primers (in excess), a thermo-stable DNA polymerase (isolated from the bacterium Thermus aquaticus (i.e., Taq DNA polymerase) and four deoxyribonucleoties. The actual technique of PCR involves repeated cycles for amplification of target DNA.

Each cycle has three stages:

1. Denaturation:

On raising the temperature to about 95° C for about one minute, the DNA gets denatured and the two strands separate.

2. Renaturation or annealing:

As the temperature of the mixture is slowly cooled to about 55° C, the primers base pair with the complementary regions flanking target DNA strands. This process is called renaturation or annealing. High concentration of primer ensures annealing between each DNA strand and the primer rather than the two strands of DNA.

3. Synthesis:

The initiation of DNA synthesis occurs at 3′-hydroxyl end of each primer. The primers are extended by joining the bases complementary to DNA strands. The synthetic process in PCR is quite comparable to the DNA replication of the leading strand.

However, the temperature has to be kept optimal as required by the enzyme DNA polymerase. For Taq DNA polymerase, the optimum temperature is around 75° C (for E. coli DNA polymerase, it is around 37° C). The reaction can be stopped by raising the temperature (to about 95° C).

The 3 stages of PCR in relation to temperature and time are depicted in Fig. 8.1. Each cycle of PCR takes about 3-5 minutes. In the normal practice, the PCR is carried out in an automated machine.

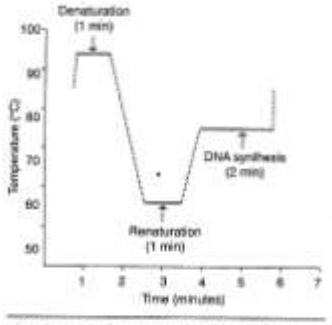


Fig. 8.1 : The three stages in each cycle of PCR in relation to temperature and time (Each cycle takes approximately 3-5 minutes).

As is evident from the Fig. 8.2 (cycle I), the new DNA strand joined to each primer is beyond the sequence that is complementary to the second primer. These new strands are referred to as long templates and they will be used in the second cycle.

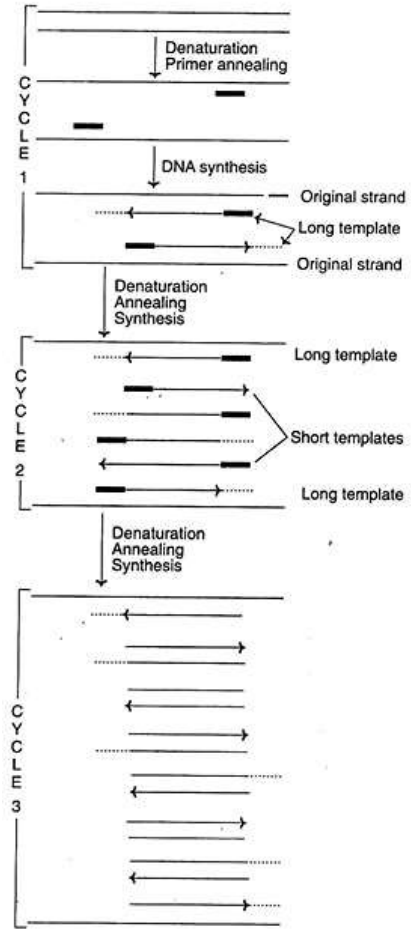


Fig. 8.2 : The polymerase chain reaction (PCR) representing the initial three cycles (— indicate primers).

For the second cycle of PCR, the DNA strands (original + newly synthesized long template) are denatured, annealed with primers and subjected to DNA synthesis. At the end of second round, long templates, and short templates (DNA strands with primer sequence at one end, and sequence complementary to the other end primer) are formed.
In the third cycle of PCR, the original DNA strands along with long and short templates are the starting materials. The technique of denaturation, renaturation and synthesis are repeated. This procedure is repeated again and again for each cycle. It is estimated that at the end of 32nd cycle of PCR, about a million-fold target DNA is synthesized (Table 8.1). The short templates possessing precisely the target DNA as double- stranded molecules accumulate.

Sources of DNA Polymerase:
In the original technique of PCR, Klenow fragment of E. coli DNA polymerase was used. This enzyme, gets denatured at higher temperature, therefore, fresh enzyme had to be added for each cycle. A breakthrough occurred with the introduction of Taq DNA polymerase from thermophilic bacterium, Thermus aquaticus. The Taq DNA polymerase is heat resistant; hence it is not necessary to freshly add this enzyme for each cycle of PCR.

Key Factors for Optimal PCR:

Primers:
Primers play a significant role in determining PCR. The primers (17-30 nucleotides) without secondary structure and without complementarity among themselves are ideal. The complementary primers can hybridize to form primer dimer and get amplified in PCR. This prevents the multiplication of target DNA.

DNA polymerase:
As already described, Taq DNA polymerase is preferred as it can withstand high temperature. In the hot-start protocol, DNA polymerase is added after the heat denaturation step of the first cycle. This avoids the extension of the mismatched primers that usually occur at low temperature.
Taq polymerase lacks proof reading exonuclease (3′-5′) activity which might contribute to errors in the products of PCR. Some other thermo-stable DNA polymerases with proof-reading activity have been identified e.g., Tma DNA polymerase from Thermotoga maritama; Pfu DNA polymerase from Pyrococcus furiosus.

Target DNA:
In general, the shorter the sequence of target DNA, the better is the efficiency of PCR. However, in recent years, amplification of DNA fragments up to 10 kb has been reported. The sequence of target DNA is also important in PCR. Thus, CC-rich regions of DNA strand hinder PCR.

Promoters and inhibitors:
Addition of proteins such as bovine serum albumin (BSA) enhances PCR by protecting the enzyme DNA polymerase. Humic acids, frequently found in archeological samples of target DNA inhibit PCR.

Variations of PCR:
The basic technique of the PCR has been described. Being a versatile technique, PCR is modified as per the specific demands of the situation. Thus, there are many variations in the original PCR; some of them are discussed, hereunder.

Nested PCR:

Sequence similarities between the target DNA and related DNA are very frequently seen. As a result of this, the primers may bind to both the DNAs and therefore even the undesired DNA also gets amplified in PCR. Use of nested primers increases the specificity of PCR, and selectively amplifies target DNA. Nested PCR is illustrated in Fig. 8.3. In the first cycle of PCR, the products are both from target DNA and undesired DNA. A second set of internal primers is now used. They will selectively bind to target DNA and amplification proceeds.

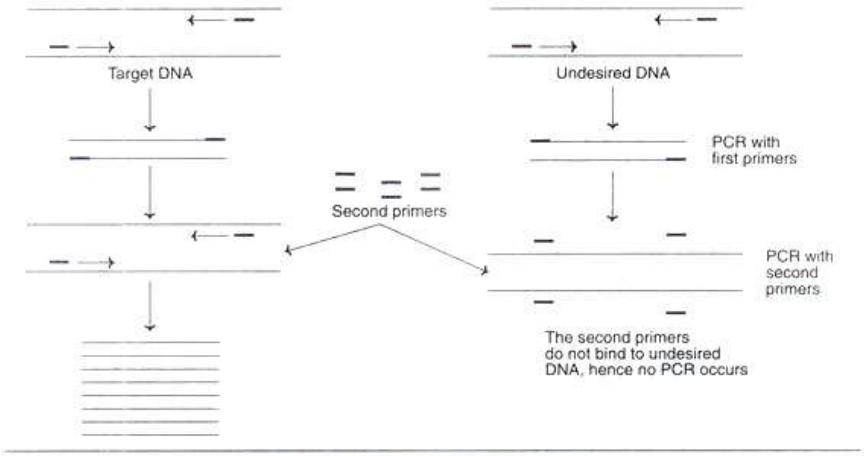


Fig. 8.3 : Nested polymerase chain reaction.

Inverse PCR:

In the inverse PCR, amplification of DNA of the unknown sequences is carried out from the known sequence (Fig. 8.4). The target DNA is cleaved with a restriction endonuclease which does not cut the known sequence but cuts the unknown sequence on either side. The DNA fragments so formed are inverted and get circularized (DNA ligase is employed as a sealing agent).

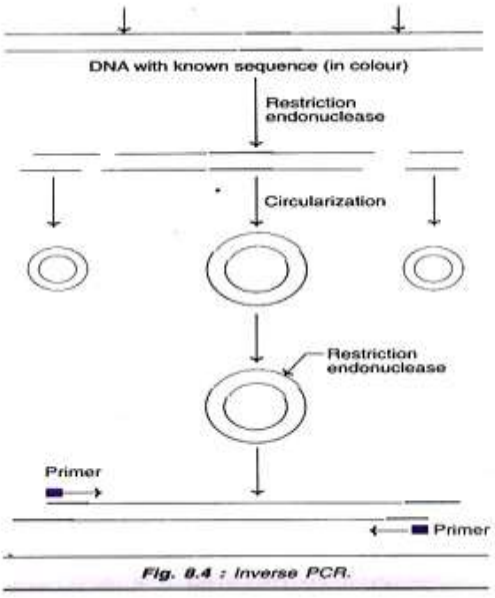


Fig. 8.4 : Inverse PCR.

The circle containing the known sequences is now cut with another restriction enzyme. This cleaves only the known sequence. The target DNA so formed contains the known sequence at both the ends with target DNA at the middle. The PCR amplification can now be carried out. It may be noted that the primers are generated in the opposite direction to the normal, since the original sequence is inverted during circularization.

Anchored PCR:

In the anchored PCR, a small sequence of nucleotides can be attached (tagged) to the target DNA i.e., the DNA is anchored. This is particularly useful when the sequence surrounding the target DNA is not known. The anchor is frequently a poly G tail to which a poly C primer is used. The anchoring can also be done by the use of adaptors. As the adaptors possess a known sequence, the primer can be chosen.

Reverse Transcription PCR:

The PCR technique can also be employed for the amplification of RNA molecules in which case it is referred to as reverse transcription — PCR (RT-PCR). For this purpose, the RNA molecule (mRNA) must be first converted to complementary DNA (cDNA) by the enzyme reverse transcriptase. The cDNA then serves as the template for PCR. Different primers can be employed for the synthesis of first strand of cDNA. These include the use of random primers, oligo dT primer and a sequence specific primer (Fig. 8.5).

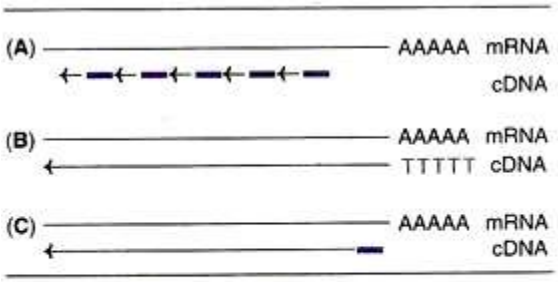


Fig. 8.5 : Synthesis of first strand of cDNA in reverse transcription-PCR with different primers (A) Random primers (B) oligo dT primer (C) Sequence specific primer (Note : The primers are shown in colour).

Asymmetric PCR:

PCR technique can also be used for the synthesis of single-stranded DNA molecules, particularly useful for DNA sequencing. In the asymmetric PCR, two primers in a ratio of 100: 1 are used. After 20-25 cycles of PCR, one primer is exhausted. The result is that in the next 5-10 PCR cycles, only single-stranded DNAs are generated.

Real-Time Quantitative PCR:

The quantification of PCR products in different cycles is not as simple as projected by theoretical considerations (Table 8.1). In practice, large variations occur. The most commonly used technique for measuring the quantity of PCR is by employing a fluorescence compound like eithidium bromide.

The principle is that the double-stranded DNA molecules bind to ethidium bromide which emit fluorescence that can be detected, and DNA quantified. The synthesis of genes by PCR and the role of PCR in site-directed mutagenesis are described elsewhere.

Random Amplified Polymorphic DNA (RAPD):

Normally, the objective of PCR is to generate defined fragments of DNA from highly specific primers. In the case of RAPD (pronounced as rapid), short oligonucleotide primers are arbitrarily selected to amplify a set of DNA fragments randomly distributed throughout the genome. This technique, random amplified polymorphic DNA is also known as arbitrarily primed PCR (AP-PCR).

The procedure of RAPD is comparable to the general technique of PCR. This method basically involves the use of a single primer at low stringency. A single short oligonucleotide (usually a 9-10 base primer) binds to many sites in the genome and the DNA fragments are amplified from them. The stringency of primer binding can be increased after a few PCR cycles. This allows the amplification of best mismatches.

RAPD can be carefully designed so that it finally yields genome- specific band patterns that are useful for comparative analysis. This is possible since genomic DNA from two different individuals often produces different amplified patterns by RAPD. Thus, a particular DNA fragment may be generated for one individual and not for the other, and this represents DNA polymorphism which can be used as a genetic marker.

RAPD is widely used by plant molecular biologists for the genetic identification of plant species. For this purpose, different combinations of nucleotides, most of them random oligonucleotide primers have been designed and are commercially available. As each random primer anneals to a different region of DNA, many different regions of loci on the DNA can be identified. RAPD is thus useful for the construction of genetic maps and as a method for genomic fingerprinting.

Limitations of RAPD:

The main problem of RAPD is associated with reproducibility. It is often difficult to obtain similar levels of primer binding in different experiments. It is therefore difficult to correlate results obtained by different research groups on RAPD.

Amplified Fragment Length Polymorphism (AFLP):

AFLP is a very Sensitive method for detecting polymorphism in the genome. It is based on the principle of restriction fragment length polymorphism and RAPD. AFLP may be appropriately regarded as a diagnostic fingerprinting technique that detects genomic restriction fragments.

In the AFLP, PCR amplification rather than Southern blotting (mostly used in RFLP) is used for the detection of restriction fragments. It may be noted that AFLP is employed to detect the presence or absence of restriction fragments, and not the lengths of these fragments. This is the major difference between AFLP and RFLP. AFLP is very widely used in plant genetics.

It has not proved useful in the mapping of animal genomes, since this technique is mainly based on the presence of high rates of substitutional variations which are not found in animals. On the other hand, substitutional variations resulting in RFLPs are more common in plants. The basic principle of AFLP involves the amplification of subsets of RFLPs using PCR (Fig. 8.6).

A genomic DNA is isolated and digested simultaneously with two different restriction endonucleases — EcoRI with a 6 base pair recognition site and Msel with a 4 base pair recognition site. These two enzymes can cleave the DNA and result in small fragments (< 1 kb) which can be amplified by PCR. For this purpose the DNA fragments are ligated with EcoRI and Msel adaptors.

These common adaptor sequences (flanking genomic sequences) serve as primer binding sites on the restriction fragments. The DNA fragments can be amplified with AFLP primers each having only one selective nucleotide. These PCR products are diluted and used as templates for the selective amplification employing two new AFLP primers that have 2 or 3 selective nucleotides. After the selective amplification by PCR, the DNA products are separated on a gel.

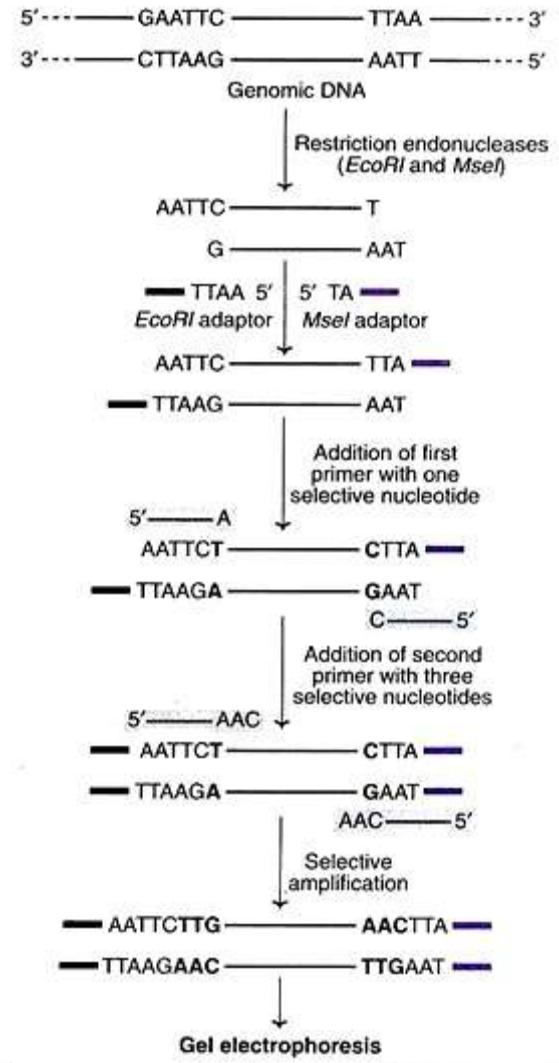


Fig. 8.6 : The technique of amplified fragment length of polymorphism (AFLP).

The resultant DNA fingerprint is identified by autoradiography. AFLP fragments represent unique positions in the genomes, and hence can be used as landmarks to bridge the gaps between genetic and physical maps of genomes. In plants, AFLP is useful to generate high density maps, and to detect genomic clones.

Rapid Amplification of cDNA Ends (RACE):

As already described (See p. 115), reverse transcription, followed by PCR (RT-PCR) results in the amplification of RNA sequences in cDNA form. But the major limitation of RT-PCR is related to incomplete DNA sequences in cDNA. This problem is solved by using the technique rapid amplification of cDNA ends. RACE is depicted in Fig. 8.7, and briefly described below.

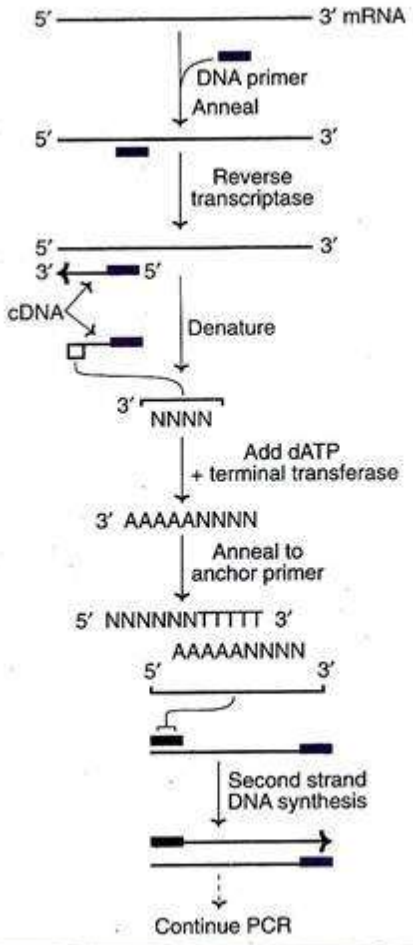


Fig. 8.7 : Rapid amplification of cDNA ends (RACE).

The target RNA is converted into a partial cDNA by extension of a DNA primer. This DNA primer was first annealed at an interval position of RNA, not too far from the 5′-end of the molecule. Now addition dATP (As) and terminal deoxynucleotidyl transferase extends the 3′-end of the cDNA.

This happens due to the addition of a series of as to the cDNA. These as series now act as the primer to anneal to the anchor primer. A second strand of DNA can be formed by extending the anchor primer. The double-stranded DNA is now ready for amplification by PCR. The above procedure described is called 5′- RACE, since it is carried out by amplification of the 5′-end of

the starting RNA. Similar protocol can be used to carry out 3′-RACE when the 3′-end RNA sequence is desired.

Limitations of RACE:

Since a specific primer is used, the specificity of amplification of RACE may not be very high. Another disadvantage is that the reverse transcriptase may not fully reach the 5′-ends of RNA, and this limits the utility of RACE. In recent years, some modifications have been done to improve RACE.

Applications

The top six applications are: (1) PCR in Clinical Diagnosis (2) PCR in DNA Sequencing (3) PCR in Gene Manipulation and Expression Studies (4) PCR in Comparative Studies of Genomes (5) PCR in Forensic Medicine and (6) PCR in Comparison with Gene Cloning.

1. PCR in Clinical Diagnosis:

The specificity and sensitivity of PCR is highly useful for the diagnosis of various diseases in humans. These include diagnosis of inherited disorders (genetic diseases), viral diseases, bacterial diseases etc. The occurrence of genetic diseases frequently identified by restriction fragment length polymorphism (RFLP) can be employed only when there is a mutation resulting in a detectable change in the length of restriction fragment. Many genetic diseases occur without the involvement of RFLP. For all such disorders, PCR technique is a real boon, as it provides direct information of DNA. This is done by amplification of DNA of the relevant region, followed by the direct analysis of PCR products.

Prenatal diagnosis of inherited diseases:

PCR is employed in the prenatal diagnosis of inherited diseases by using chorionic villus samples or cells from amniocentesis. Thus, diseases like sickle-cell anemia, p-thalassemia and phenylketonuria can be detected by PCR in these samples.

Diagnosis of retroviral infections:

PCR from cDNA is a valuable tool for diagnosis and monitoring of retroviral infections, e.g., HIV infection.

Diagnosis of bacterial infections:

PCR is used for the detection of bacterial infection e.g., tuberculosis by Mycobacterium tuberculosis.

Diagnosis of cancers:

Several virally-induced cancers (e.g., cervical cancer caused by human papilloma virus) can be detected by PCR. Further, some cancers which occur due to chromosomal translocation (chromosome 14 and 18 in follicular lymphoma) involving known genes are identified by PCR.

PCR in sex determination of embryos:

Sex of human and livestock embryos fertilized in vitro, can be determined by PCR, by using primers and DNA probes specific for sex chromosomes. Further, this technique is also useful to detect sex — linked disorders in fertilized embryos.

2. PCR in DNA Sequencing:

As the PCR technique is much simpler and quicker to amplify the DNA, it is conveniently used for sequencing. For this purpose, single-strands of DNA are required. In asymmetric PCR, preferential amplification of a single-strand is carried out. In another method, strand removal can

be achieved by digesting one strand (usually done by exonuclease by its action on 5′-phosphorylated strand).

3. PCR in Gene Manipulation and Expression Studies:

The advantage with PCR is that the primers need not have complementary sequences for the target DNA. Therefore, the sequence of nucleotides in a piece of the gene (target DNA) can be manipulated and amplified by PCR.

By using this method, coding sequence can be altered (thereby changing amino acids) to synthesize protein of interest. Further, gene manipulations are important in understanding the effects of promoters, initiators etc., in gene expression.

PCR is important in the study of mRNAs, the products of gene expression. This is carried out by reverse transcription — PCR.

4. PCR in Comparative Studies of Genomes:

The differences in the genomes of two organisms can be measured by PCR with random primers. The products are separated by electrophoresis for comparative identification. Two genomes from closely related organisms are expected to yield more similar bands. For more details, refer the technique random amplified polymorphic DNA.

PCR is very important in the study evolutionary biology, more specifically referred to as phylogenetic. As a technique which can amplify even minute quantities of DNA from any source (hair, mummified tissues, bone, or any fossilized material), PCR has revolutionized the studies in palaentology and archaelogy. The movie 'Jurassic Park' has created public awareness of the potential applications of PCR!

5. PCR in Forensic Medicine:

A single molecule of DNA from any source (blood strains, hair, semen etc.) of an individual is adequate for amplification by PCR. Thus, PCR is very important for identification of criminals.

The reader may refer DNA finger printing technique described elsewhere.

6. PCR in Comparison with Gene Cloning:

PCR has several advantages over the traditional gene cloning techniques .These include better efficiency, minute quantities of starting material (DNA), cost-effectiveness, minimal technical skill, time factor etc. In due course of time, PCR may take over most of the applications of gene cloning.

DNA foot and finger printing

DNA footprinting is a molecular technique used to identify the specific DNA sequence (binding site) that binds to a protein. This technique mainly used to identify the tran script ion factors which bind to promoter, enhancer or silencer region of gene to regulate its expression. Therefore the regulation of tran script ion of a gene can be studied using this method.

Tran script ion is a process where the DNA is converted into RNA in a cell nucleus. Initiation of tran script ion takes place when the enzyme RNA polymerase binds to a gene sequence known as promoter sequence. DNA footprinting can be used to identify RNA polymerase interacting DNA sequence.

Principle: In this technique, nucleases like DNAse I is used which will degrade DNA molecule. Nucleases cannot degrade DNA if it is bounded by a protein. Thus that region is protected from degradation by nucleases. This protected DNA region is called the foot print.

DNA footprinting Procedure:

1. DNA fragment thought to contain protein binding sequence is extracted, amplified and labelled at one end of the double helix using polymerase chain reaction technique.
2. Labelled DNA fragments with DNA binding protein and cleavage agent are mixed in a test tube.
3. In another test tube labelled DNA fragments are mixed with cleavage agents without DNA binding protein. This is used as standard to compare the results.
4. Cleavage agent cuts the DNA fragment present in the both test tubes but no cuts are made at the specific region of DNA where proteins are bound. Protein has protected the DNA binding site from cleavage agent.
5. DNA fragments are separated by polyacrylamide gel electrophoresis and visualized using autoradiogram.
6. When compared with the standard missing band (footprint) indicates the protein binding specific DNA sequence.

Cleavage Agent:

Cleavage agents used in DNA footprinting are

1. DNase I:

DNae I is a double strand endonuclease enzyme. DNase I cleaves the phosphodiester bond present in the DNA. The enzyme action can be controlled by EDTA solution. The action of the enzyme is DNA structure and sequence specific, resulting in an uneven ladder. This can also affect the precision of predicting protein's specific DNA sequence.

2. Hydroxyl radicals:

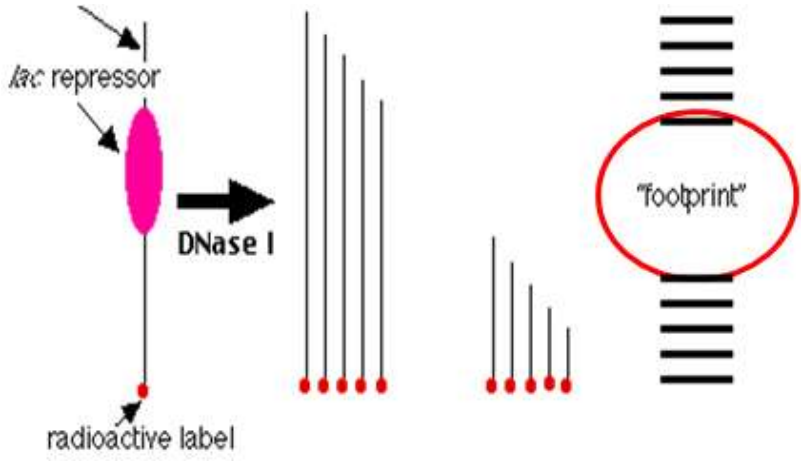
Hydroxyl radicals are produced from a reaction where iron salt is reduced with hydrogen peroxide to form free hydroxyl molecule. The free hydroxyl molecule cleaves the DNA fragment. Hydroxyl radicals are independent of DNA sequence for their action, therefore form evenly distributed ladder. But the reaction rates of these radicals are very slow therefore it requires more time to cleave the DNA fragments.

3. Ultraviolet radiation:

Ultraviolet radiations are used to excite the nucleic acid and this may lead to damaged DNA fragments.

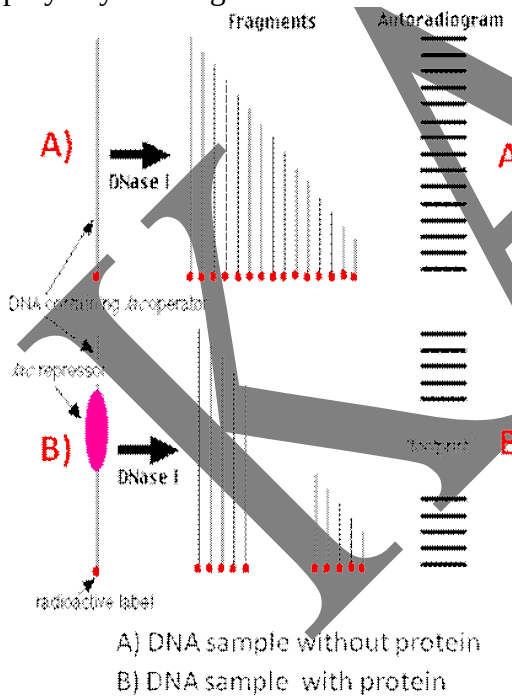
Applications of DNA Footprinting:

1. DNA footprinting can be used to determine the sequence specific DNA-binding protein site.
2. Interaction between protein and DNA can be studied using this technique both in-vivo and ex-vivo of a cell.
3. Tran script ional regulations can be studied using DNA footprinting technique.
4. Promoter, enhancer and silencer sequence of a gene can be identified
5. Scientists and researchers can use this technique to identify the functional genes present in the large genome of human.



1.Radioactive 5' end labeling of the DNA suspected to contain one or more protein binding sites
2.Two DNA samples; one incubated with suspected protein and other without the protein
3.The DNA is treated with a nuclease such as DNAse I, that digests only unprotected DNA

DNAse I is used under specific digestion condition to obtain one cut or hit per molecule, resulting in a complete base ladder (one base difference) when electrophoresed in 6-8% polyacrylamide gel.



A) DNA sample without protein
B) DNA sample with protein

4.The resulting products are separated on a Polyacrylamide gel electrophoresis (PAGE) In DNA sample with protein, protein binding regions are protected from degradation by DNAse I
5.X-ray film exposure and autoradiography.

Comparison of both samples reveals foot prints or protein binding sites. In the figure

- **DNA Sample A without protein:** consistent degradation by DNAse I resulting in a continuous ladder
- **DNA Sample B with protein (lac repressor):** Interrupted degradation by DNAse I as protein or lac repressor bound regions are protected from cleavage by DNAse I. This protected DNA region is called the "DNA foot print"

DNA Fingerprinting

DNA fingerprinting is based on sequence polymorphisms, slight sequence differences (usually single base-pair changes) between individuals, 1 bp in every 1,000 bp, on average.

Each difference from the prototype human genome, sequence (the first one obtained) occurs in some fraction of the human population; every individual has some differences.

Some of the sequence changes affect recognition sites for restriction enzymes, resulting in variation in the size of DNA fragments produced by digestion with a particular restriction enzyme.

These variations are restriction fragment length polymorphisms (RFLPs). The detection of RFLPs relies on a specialized hybridization procedure called Southern blotting. DNA fragments from digestion of genomic DNA by restriction endonucleases are separated by size electrophoretic-ally, denatured by soaking the agarose gel in alkali, and then blotted onto a nylon membrane to reproduce the distribution of fragments in the gel.

The membrane is immersed in a solution containing a radioactively labelled DNA probe. A probe for a sequence that is repeated several times in the human genome generally identifies a few of the thousands of DNA fragments generated when the human genome is digested with a restriction endonuclease. Autoradiography reveals the fragments to which the probe hybridizes.

The genomic DNA sequences used in these tests are generally regions containing repetitive DNA (short sequences repeated thousands of times in tandem), which are common in the genomes of higher eukaryotes. The number of repeated units in these DNA regions varies among individuals (except between identical twins).

With a suitable probe, the pattern of bands produced by DNA fingerprinting is distinctive for each individual. Combining the use of several probes makes the test so selective that it can positively identify a single individual in the entire human population. However, the Southern blot procedure requires relatively fresh DNA samples and larger amounts of DNA than are generally present at a crime scene.

RFLP analysis sensitivity is augmented by using PCR to amplify vanishingly small amounts of DNA. This allows investigators to obtain DNA fingerprints from a single hair follicle, a drop of blood, a small semen sample from a rape victim, or samples that might be months or even many years old.

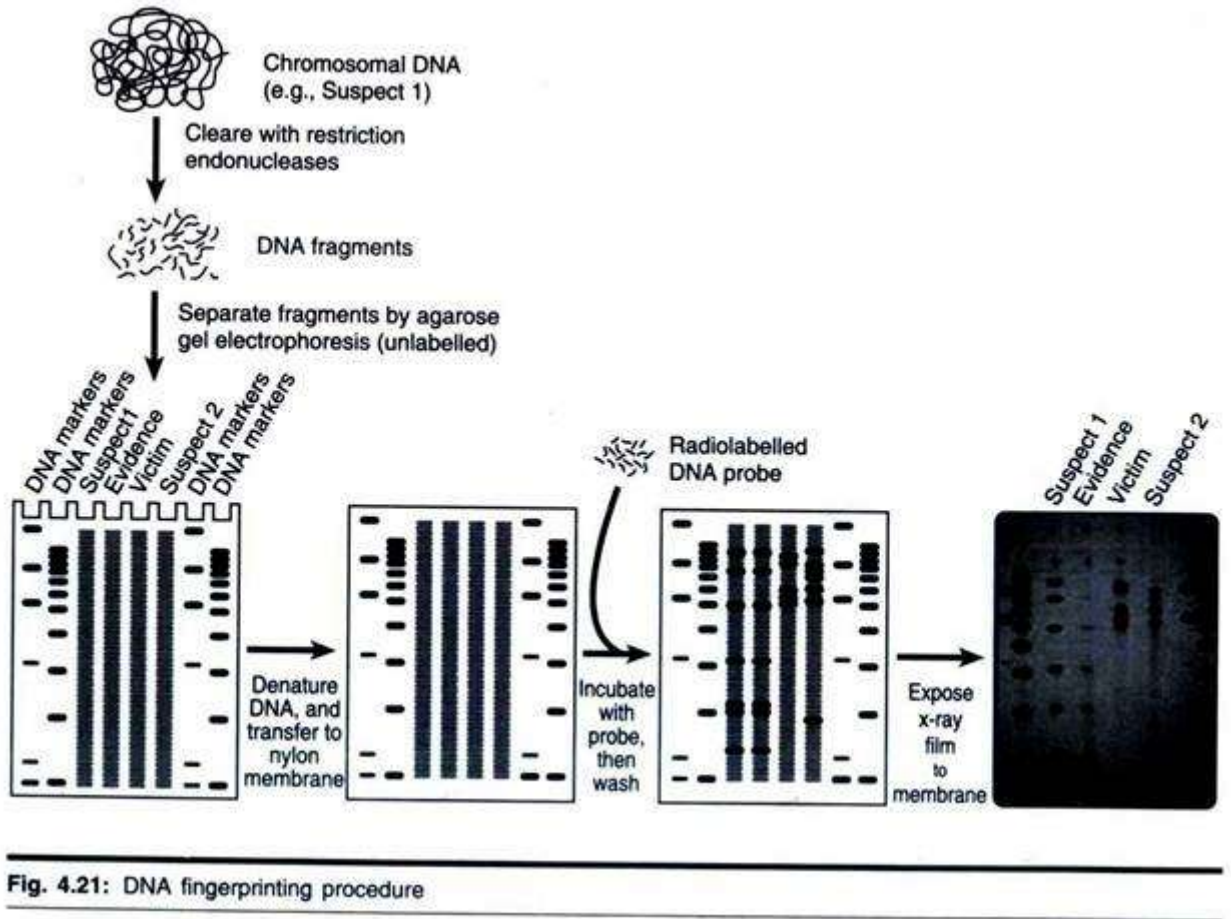


Fig. 4.21: DNA fingerprinting procedure

These methods are proving decisive in court cases worldwide. In the example in Fig. 4.21, the DNA from a semen sample obtained from a rape and murder victim was compared with DNA samples from the victim and two suspects. Each sample was cleaved into fragments and separated by gel electrophoresis. Radioactive DNA probes were used to identify a small subset of fragments that contained sequences complementary to the probe.

The sizes of the identified fragments varied from one individual to the next, as seen here in the different patterns for the three individuals (victim and two suspects) tested. One suspect's DNA exhibits a banding pattern identical to that of a semen sample taken from the victim. This test used a single probe, but three or four different probes would be used (in separate experiments) to achieve an unambiguous positive identification.

Such results have been used to both convict and acquit suspects and, in other cases, to establish paternity with an extraordinary degree of certainty. The impact of these procedures on court cases will continue to grow as societies agree on the standards and as formal methods become widely established in forensic laboratories. Even decades-old murder mysteries can be solved: in 1996, DNA fingerprinting helped to confirm the identification of the bones of the last Russian czar and his family, who were assassinated in 1918.

This is also known as 'DNA PROFILING' o 'DNA TYPING'. DNA fingerprinting is a technique to identify a person on the basis of his/her DNA specificity.

The practice of using thumbs impression of a person, as an identifying mark is very well known since long.

The study of finger, palm and sole prints is called dermatoglyphics and it has been a subject of human interest.

But, the concept of DNA fingerprinting is totally a new approach in the field of molecular biology. Sir Alec Jeffreys (1985-86) invented the DNA fingerprinting technique at Leicester University, United Kingdom.

Meaning:

DNA of an individual carries some specific sequence of bases, which do not carry any information for protein synthesis. Such nucleotide base sequences are repeated many times and are found in many places throughout the length of DNA. The number of repeats is very specific in each individual. The tandem repeats of short sequences are called 'mini satellites' or 'variable number tandem repeats' (VNTRs). Such repeats are used as genetic markers in personal identity.

Technique:

1. The first step is to obtain DNA sample of the individual in question.
2. DNA is also isolated from bloodstains, semen stains or hair root from the body of the victim or from victim's cloth even after many hours of any criminal offence. Even it can be obtained from vaginal swabs of rape victims. The amount of DNA needed for developing fingerprints is very small, only a few nanograms.
3. The DNA is digested with a suitable restriction endonuclease enzyme, which cuts them into fragments.
4. The fragments are subjected to gel electrophoresis by which the fragments are separated according to their size.
5. The separated fragments are copied onto a nitrocellulose filter membrane by Southern blotting technique.
6. Special DNA probes are prepared in the laboratory and made radioactive by labeling with radioactive isotopes. These probes contain repeated sequences of bases complimentary to those on mini satellites.
7. The DNA on the nitrocellulose filter membrane is hybridized with the radioactive probes and the free probes are washed off.
8. The bands to which the radioactive probes have been hybridized are detected through autoradiography. This is a technique where an X-ray film is exposed to the nitrocellulose membrane to mark the places where the radioactive DNA probes have bound to the DNA fragments. These places are marked as dark bands when X-ray film is exposed.
9. The dark bands on the X-ray film represent the DNA fingerprints or DNA profiles.
10. Comparison is made between the banding pattern of collected DNA sample and suspected human subject to confirm the criminal with hundred percent accuracy (Fig.5.24).

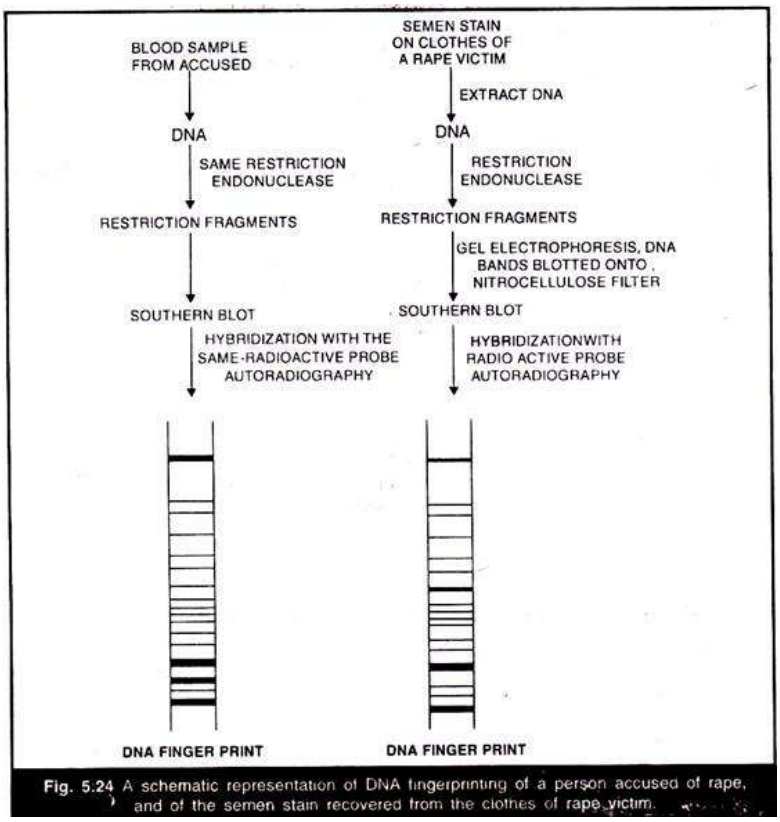


Fig. 5.24 A schematic representation of DNA fingerprinting of a person accused of rape, and of the semen stain recovered from the clothes of rape victim.

Significance:

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

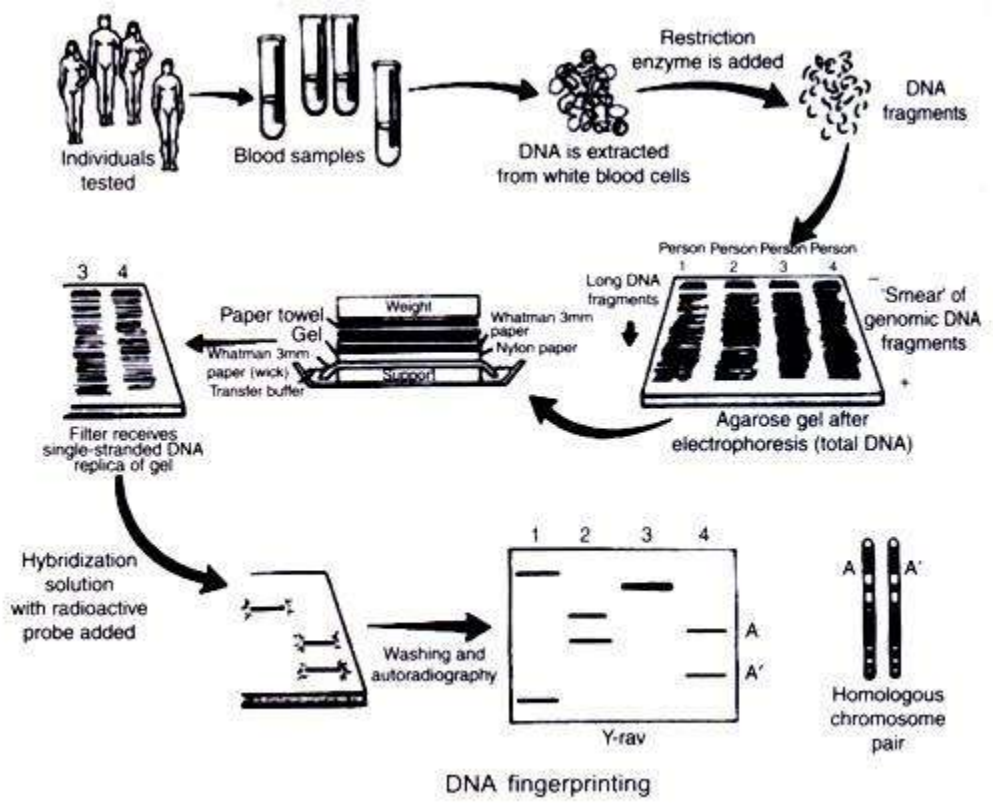
DNA Fingerprinting Technique:

The main types of DNA fingerprinting methods in use at this time are RFLP, PGR, Amp FLP and STR.

(A) RFLP:

Restriction fragment length polymorphism (RFLP) analyzes the length of the strands of the DNA molecules with repeating base pair patterns. DNA molecules are long strands found tightly wound in chromosomes which are contained in the nucleus of each human cell.

With each DNA strand are numbers of genes that determine the particular characteristics of an individual. While about 5% of the gene compositions on DNA contain this type of genetic information, the other 95% do not. However, of the 95%, these non-coding genes contain identifiable repetitive sequences of base pairs, which are known as VNTR.



DNA fingerprinting

The restriction fragment length polymorphism analysis is used to detect the repeated sequences by determining a specific pattern to the VNTR, which becomes the person's DNA fingerprint. Inclusions are isolation of DNA, digestion of DNA by restriction endonucleases, separation of DNA fragments by electrophoresis, transferring (blotting) of separated DNA fragments to synthetic membranes.

(b) PCR:

PGR (Polymerase chain reaction) PGR analysis amplifies the DNA molecules using a smaller sample. The PGR was found to be useful in identifying DNA fingerprints in criminal matters on the forensic front. In paternity tests, it requires less amounts of DNA because it makes identical copies of the DNA sample. The PGR analysis amplified isolated regions on the strands of the DNA under examination, therefore, it was not as discriminating as the RFLP.

(c) AmpFLP:

AmpFLP (Amplified fragment length polymorphism) AmpFLP came into vogue in the 90's and is still popular in the smaller countries involved in the process of DNA fingerprinting. It is relatively less complicated operation and has the cost-effectiveness of the procedure.

By using the PGR analysis to amplify the minisatellite loci of the human cell, this method proved quicker in recovery than the RFLP. There are issues of bunching of the VTRN's, causing misidentifications in the process due to the use of gel in its analysis phase.

(D) STR:

The system most widely form of DNA fingerprinting STR is the (Short tandem repeat) methodology for extracting DNA. This system is based on the features of PGR, as it utilizes specific areas that have short sequential repeat DNA.

The STR analyzes how many times base pairs repeat themselves on a particular location on a strand of DNA. The DNA comparisons can match the possibilities into an almost endless range; therefore, it is the big advantage in this method.

DNA fingerprinting has been extremely successful for use in the personal identification of criminal suspects. DNA testing for ethnicity, identification of the decreased, as well as court-approved paternity tests. However, Still DNA poses issues as the VNTRs are not evenly distributed in all people as they are inherited. Further, there is still the imperfect human element as the final voice in the administration of all DNA fingerprinting procedures.

DNA Fingerprinting Application:

(a) Forensic Analyses on Animals:

Medigenomix laboratory offers a wide variety of methods unequivocally assigning biological traces to individuals. Medigenomix's genotyping services include identity testing and forensic DNA trace analysis. DNA fingerprinting is a state-of-the-art method.

Mostly police laboratories process the sample of human origin. Medigenomix worked on a case where a bone chewed by a dog and left at a crime scene, convicted the owner of the dogs as a thief. With currently microsatellite markers available for many different species, therefore, the laboratory can identify dogs, cats, horses, cattle, pigs as well as deer, fox and other wild animals.

(b) Ancient DNA:

Medigenomix is also the scientists have succeeded in drawing up DNA- fingerprints of 30,000 years old mammoth hones from Alaska and Siberia by microsatellite analysis. They have excellent results.

(c) Paternity Testing:

PGR is (Polymerase Chain Reaction) produces the genetic fingerprint, which is highly specific for each individual. Genome of every single individual is the combination of the gemones from both parents; therefore the DNA profile of an individual is a combined pattern of parental genetic markers.

A reliable assignment of paternity for each individual permits the comparative analysis of a specific set of these microsatellite markers permits. Specimens for paternity testing are usually taken from cells inside the mouth. Biological samples for trace analyses can be selected from Blood, sperm, skin and even excrement provide.

(d) DNA Profile in Credit Card Format:

Identification of victims of plane accidents, explosions, terrorist attacks or fire disasters in tunnels is done by Personal DNA profiling Medigenomix initiated its M-Card two years ago. It offers the opportunity for inviduals to have their unique DNA-profile in the format of a credit card.

The M-Card profile has 99.9999% accuracy in identification of any individual. Stored in a safe place, the M-Card provides identification after an accident when the victim cannot be identified on the basis of morphological features.

(e) General Information and Data Security:

In the M-Card profile, the DNA regions used for individual identification are specific isolated genetic loci in the non-coding regions of the genomic DNA. Here no functional genes are encoded; therefore, it is not possible to derive any information concerning potential genetic diseases or personal features from these results.

Hybridisation probes

A probe is a small, fluorescently or radioactively labeled DNA molecule that is used to locate similar or complementary sequences among a long stretch of DNA molecule or bacterial colonies such as genomic or cDNA libraries or in a genome. Such DNA probes are used in hybridization experiments such as Southern hybridization to detect certain specific sequences, which are complementary to the probes. Since the probe is labeled with a fluorescent dye or radioactive isotopes of phosphorous, its binding to specific sequences can be detected. DNA probes labeled with radioactive isotopes or fluorescent dyes can be used for the screening of transformed colonies having the correct recombinant plasmid by Southern hybridization.

Radiolabelled

A DNA molecule is usually labeled by incorporating nucleotides that carry a radioactive isotope of phosphorus, 32P (Figure a). Several methods are available:

Nick translation. Most purified samples of DNA contain some nicked molecules, however carefully the preparation has been carried out, which means that DNA polymerase I is able to attach to the DNA and catalyze a strand replacement reaction (Figure). This reaction requires a supply of nucleotides: if one of these is radioactively labeled, the DNA molecule will itself become labeled. Nick translation can be used to label any DNA molecule but might under some circumstances also cause DNA cleavage.

End filling is a gentler method than nick translation and rarely causes breakage of the DNA, but unfortunately can only be used to label DNA molecules that have sticky ends. The enzyme used is the Klenow fragment which "fills in" a sticky end by synthesizing the complementary strand (Figure). As with nick translation, if the end filling reaction is carried out in the presence of labeled nucleotides, the DNA becomes labeled.

Random priming results in a probe with higher activity and therefore able to detect smaller amounts of membrane-bound DNA. The denatured DNA is mixed with a set of hexameric oligonucleotides of random sequence. By chance, these random hexamers will contain a few molecules that will base pair with the probe and prime new DNA synthesis. The Klenow fragment is used as this enzyme lacks the nuclease activity of DNA polymerase I (p. 48) and so only fills in the gaps between adjacent primers (Figure). Labeled nucleotides are incorporated into the new DNA that is synthesized.

After hybridization, the location of the bound probe is detected by **autoradiography**.A sheet of X-ray-sensitive photographic film is placed over the membrane. The radioactive DNA exposes the film, which is developed to reveal the positions of the colonies or plaques to which the probe has hybridized

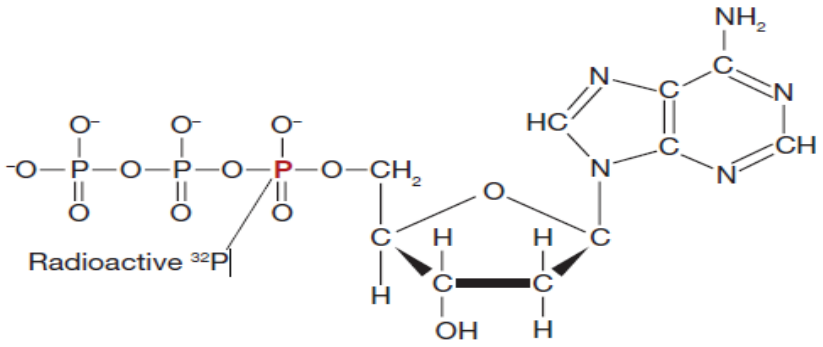


Fig: The structure of □-32P-deoxyadenosine triphosphate ([□-32P]dATP).

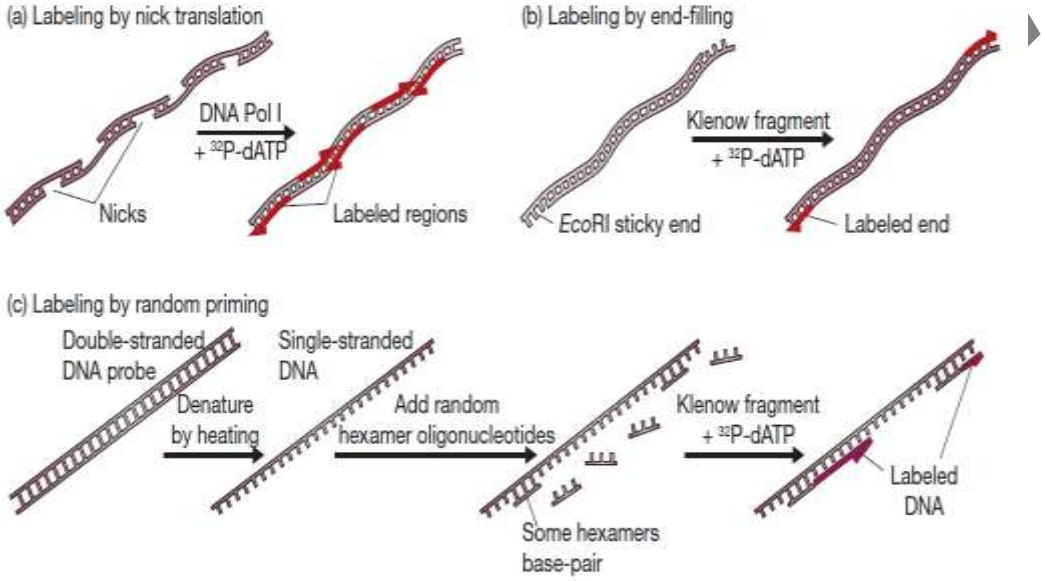


Fig: Methods for labeling DNA.

Non-radioalabelled

Radioactive labeling methods are starting to fall out of favor, partly because of the hazard to the researcher and partly because of the problems associated with disposal of radioactive waste. As an alternative, the hybridization probe can be labeled in a non-radioactive manner. A number of methods have been developed, two of which are
illustrated in Figure 8.12. The first makes use of deoxyuridine triphosphate (dUTP) nucleotides modified by reaction with **biotin**, an organic molecule that has a high affinity for a protein called **avidin**. After hybridization the positions of the bound biotinylated probe can be determined by washing with avidin coupled to a fluorescent marker
(Figure a). This method is as sensitive as radioactive probing and is becoming increasingly popular.

The same is true for a second procedure for non-radioactive hybridization probing, in which the probe DNA is complexed with the enzyme **horseradish peroxidase**, and is detected through the enzyme's ability to degrade luminol with the emission of chemiluminescence (Figure b). The signal can be recorded on normal photographic film in a manner analogous to autoradiography.

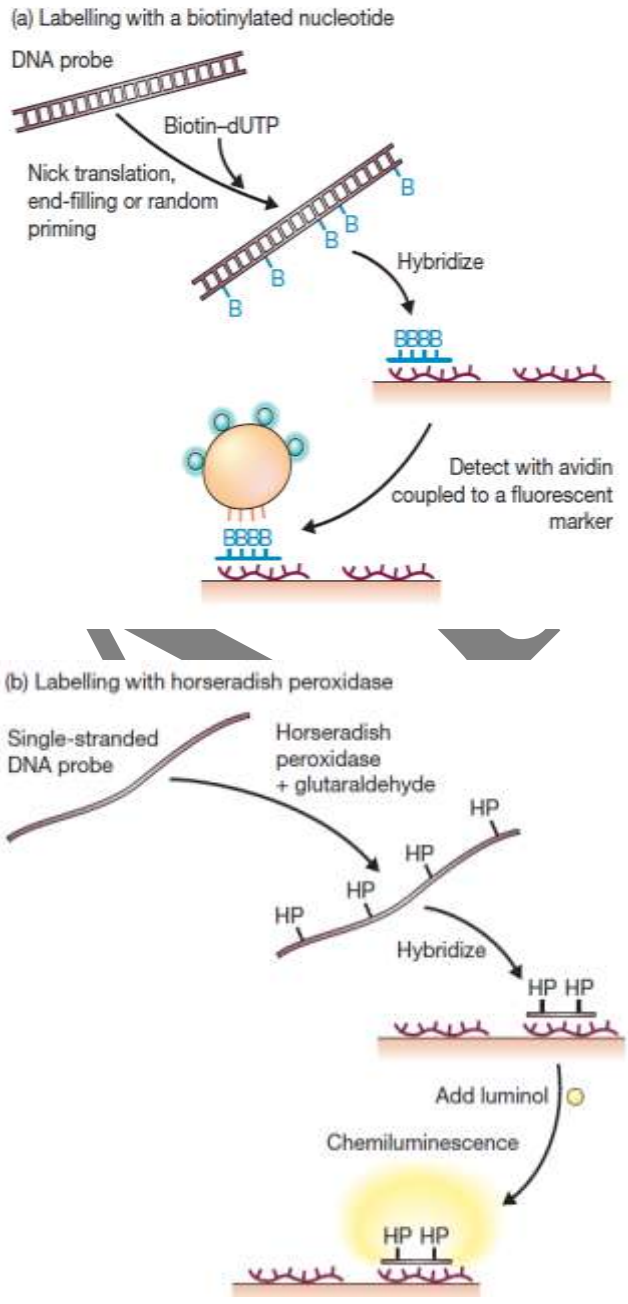


Fig: Methods for the non-radioactive labelling of DNA probes.

Examples of the practical use of hybridization probing

- Oligonucleotide probes for genes whose translation products have been characterized
- Heterologous probing allows related genes to be identified.

Hybridisation techniques

Any two single-stranded nucleic acid molecules will attempt to base pair with one another under appropriate conditions. In most cases, the hybrid structures thus formed will be very unstable since the total number of H-bonds that formed is very low. However, if there is **significant sequence complementarity** between the two strands, **stable hybrids** will be formed. This phenomenon of **nucleic acid hybridization** can be used to identify a particular recombinant if a suitable probe, complementary to the sequence sought, is available.

Southern blotting

Experimentally, the gene library to be screened, or the DNA bands separated by electrophoresis on an agarose gel, is transferred to a nitrocellulose or nylon filters by blotting. These are then processed to release DNA from the bacteria and/or the phage. These are then denatured to separate the complementary strands and are immobilized on to the membrane, in the position occupied by the original clone and in a way that leaves the bases free to interact with the complementary molecules. The probe molecules are then labeled (traditionally with radioactive nucleotides but now more commonly with chromogenic or chemiluminescent labels). Probe and filter are then incubated under conditions that promote hybridization after which the unbound probe is washed off from the filter and the specifically bound probe is visualized. Positive signals reveal the position (and thus identity) of those recombinants carrying the sequences related to the probe.

A fragment of DNA in a genome can also be detected by **Southern hybridization**. This technique was originally devised by Edward Southern in 1975n to identify specific DNA fragments on an agarose gel after the separation by electrophoresis. The technique involves the isolation of the genomic DNA with a suitable procedure. The genomic DNA is digested with a suitable restriction enzyme or with a mixture of different restriction enzymes to cut the long DNA molecule into fragments. Restriction-digested genomic DNA is separated by electrophoresis and the DNA separated on the gel is then transferred to a membrane—a nitrocellulose or nylon membrane—with a process called **blotting**, which is driven by capillary action. The blotting can also be carried out electrophoretically, and then it is known as **electroblotting**. There is another equally effective blotting method known as **vacuum blotting**. The DNA fragments transferred to the membrane are immobilized to its surface by heat or UV mediated cross-linking. Now the membrane is hybridized with a 'labeled' segment of the gene understudy called a probe (the label may be radioactive or fluorescent chemical labeling). When the hybridized filter is exposed to x-ray film, only the band with the labeled probe hybridized to it will be shown on the film. Southern hybridization allows the precise localization of a given DNA sequence in a genome, once a restriction map has been constructed. If the probe is labeled with a fluorescent chemical then the fluorescent band can be visualized after illuminating the nylon or nitrocellulose membrane with UV light after the process of blotting.

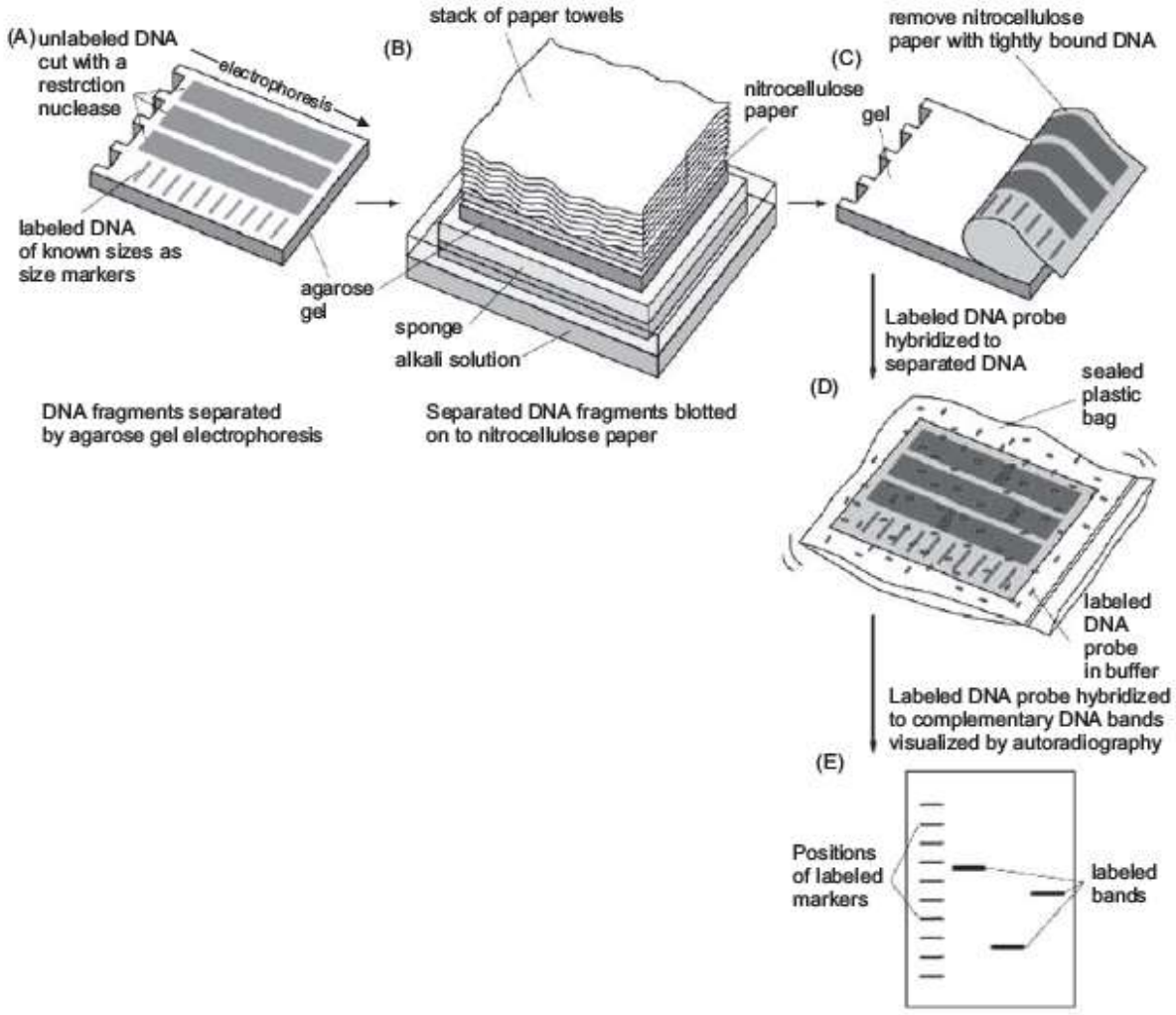


FIG: Diagram representation of the various steps in the technique of Southern hybridization.

Since the DNA bands on the membrane will have the same pattern as that present in the gel, the position of a labeled band on the membrane can be traced to the same corresponding position in the gel. There are a number of variations of Southern hybridization or Southern blotting suited for specific types of experiments. Some of them are dot blots, colony hybridization, slot blots, etc., which are comparatively easier. Similar to Southern hybridization there are altered forms of molecular hybridization techniques developed for RNA and proteins. There are the techniques developed in which RNA is hybridized with its complementary DNA sequences or a specific protein such as antibody hybridized to another protein such as its ligand or antigen. Since hybridization of DNA with another DNA molecule is called Southern hybridization or blotting, hybridization between RNA and DNA is called **Northern hybridization** and protein-protein

hybridization such as antigen-antibody binding or protein-ligand binding is called **Western hybridization**.

Northern blotting

-Detecting the presence of a transcript and determining its nucleotide sequence

Before studying the more sophisticated techniques for RNA analysis, we must consider the methods used to obtain basic information about a transcript. The first of these methods is **northern hybridization**, the RNA equivalent of Southern hybridization which is used to measure the length of a transcript. An RNA extract is electrophoresed in an agarose gel, using a denaturing electrophoresis buffer (e.g., one containing formaldehyde) to ensure that the RNAs do not form inter- or intramolecular base pairs, as base pairing would affect the rate at which the molecules migrate through the gel. After electrophoresis, the gel is blotted onto a nylon or nitrocellulose membrane, and hybridized with a labeled probe (Figure). If the probe is a cloned gene, the band that appears in the autoradiograph is the transcript of that gene. The size of the transcript can be determined from its position within the gel, and if RNA from different tissues is run in different lanes of the gel, then the possibility that the gene is differentially expressed can be examined. Once a transcript has been identified, cDNA synthesis can be used to convert it into a double-stranded DNA copy, which can be cloned and sequenced. Comparison between the sequence of the cDNA and the sequence of its gene will reveal the positions of introns and possibly the start and end points of the transcript. For this to be possible, the cDNA must be a full length copy of the mRNA from which it is derived. The 3′ end of the transcript will usually be represented in the cDNA, because most methods for cDNA synthesis begin with a primer that anneals to the poly(A) tail of the mRNA, which means that the 3′ end is the first part to be copied (see Figure). The Cdna synthesis might not, however, continue all the way to the 5′ end of the transcript, especially if the RNA is more than a few hundred nucleotides in length. Premature termination of cDNA synthesis will result in a cDNA that is not a full length copy of its transcript, and whose 3′ end does not correspond to the true 5′ end of the mRNA (Figure). If this is the case, then the start point for transcription cannot be identified from the sequence of the cDNA. It will also be impossible to map the positions of any introns that are located near the start of the gene.

Northern blotting uses mRNA as marker. The southern blotting can be extended to detect a specific RNA molecule from a mixture of RNAs fractioned on a gel. This technique is called Northern Blotting to contrast it with the Southern's technique for DNA analysis.

The fractioned RNA is blotted on to a membrane and probed in the same way as for Southern blotting. One application of Northern analysis is to determine whether specific gene is transcribed in certain tissue or under certain environmental conditions. RNAse is extracted from appropriate cell sample, then electro-phoresed, blotted and probed with the cloned gene in question. A positive signal shows presence of the transcript (Fig. 23.3).

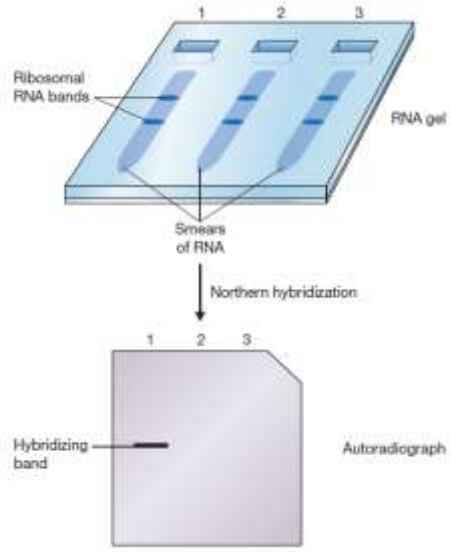


Fig: Northern hybridization. Three RNA extracts from different tissues have been electrophoresed in an agarose gel. The extracts are made up of many RNAs of different lengths so each gives a smear of RNA, but two distinct bands are seen, one for each of the abundant ribosomal RNAs. The sizes of these rRNAs are known (e.g. 4718 and 1874 nucleotides in mammals), so they can be used as internal size markers. The gel is transferred to a membrane, probed with a cloned gene, and the results visualized, for example by autoradiography if the probe has been radioactively labeled. Only lane 1 gives a band, showing that the cloned gene is expressed only in the tissue from which this RNA extract was obtained.

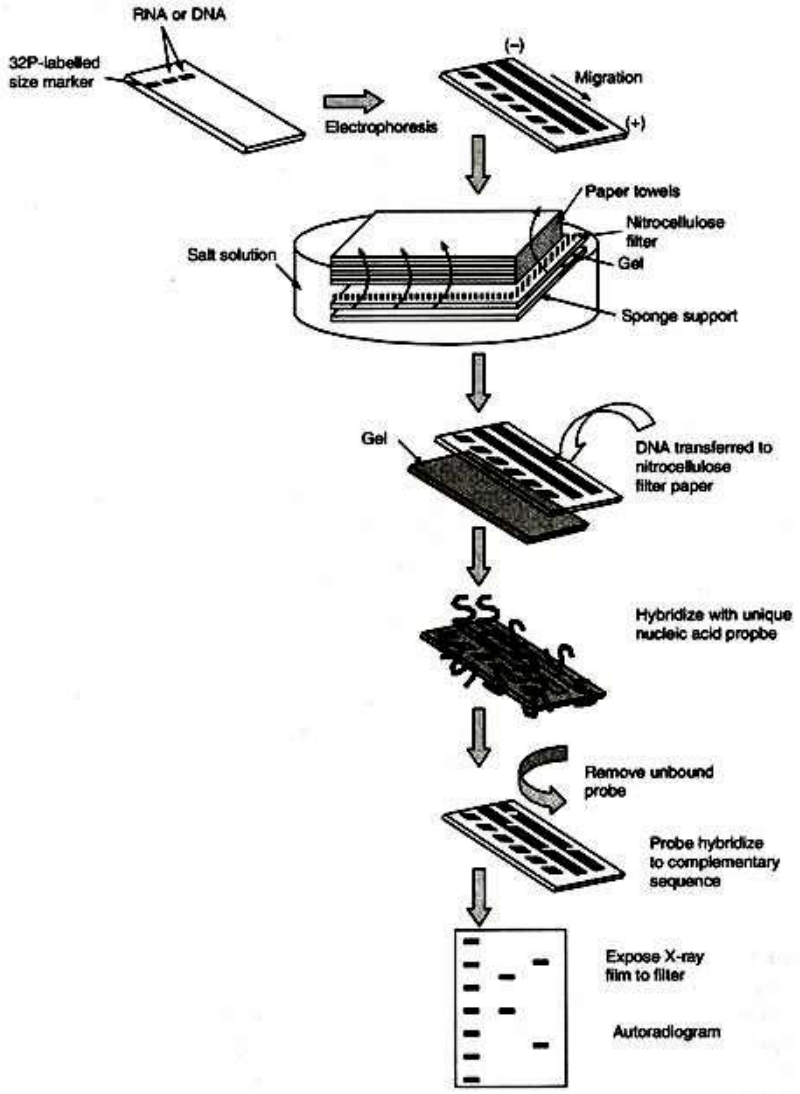


Fig. 23.3. Blotting technique (DNA/RNA is used termed as Southern/Northern technique).

Western Blotting

Western blotting uses protein as marker compound. Western blotting (also called immune-blotting because an antibody is used to specifically detect its antigen) and is now a routine technique for protein analysis. Towbin et al. (1979) developed the technique to detect the proteins of a particular specificity. The specificity of the antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein mixture. Western blotting is commonly used to positively identify a specific protein in a complex mixture and to obtain qualitative and semi-quantitative data about that protein. When the transferred gene expresses the protein in alien cell, then it is detected through this technique.

The first step in a Western blotting procedure is to separate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane.

Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexes with an enzyme-labelled antibody as a probe.

An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorimetric detection, respectively. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a byproduct. The light output can be captured using film, a CCD camera or a phosphor imager that is designed for chemiluminescent detection.

Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane.

Detailed procedures for detection of a Western blot vary widely. One common variation involves direct vs. indirect detection as shown in Figure 23.4. With the direct detection method, the primary antibody that is used to detect an antigen on the blot is also labeled with an enzyme or fluorescent dye.

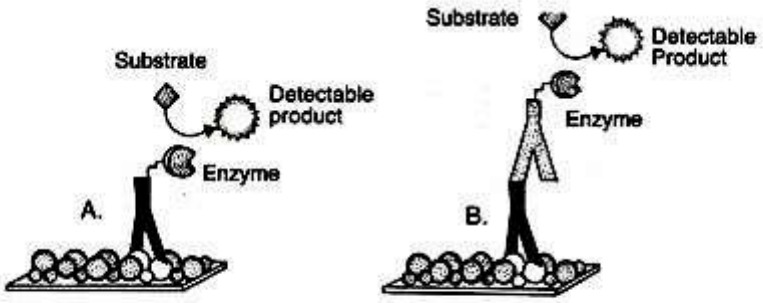


Fig. 23.4. A. In the direct detection method, labeled primary antibody binds to antigen on the membrane and reacts with substrate, creating a detectable signal. B. In the indirect detection method, unlabeled primary antibody binds to the antigen. Then, a labeled secondary antibody binds to the primary antibody and reacts with the substrate.

In the indirect detection method, a primary antibody is added first to bind to the antigen. This is followed by a labeled secondary antibody that is directed against the primary antibody. Labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish per-oxidase or alkaline phosphatase. The indirect method offers many advantages over the direct method.

Site-directed mutagenesis

Any heritable change in the genome is commonly called a **mutation**. Biochemically, it is a chemical change or alteration in a nitrogen base of a DNA sequence resulting in the production of a defective protein or a truncated protein, which is not functional. These altered proteins can cause serious problems in metabolism leading to changes in the morphology and physiology of the organism. Mutations, in most cases, are spontaneous and may not be dangerous. Even though the natural mutations are spontaneous and rare, biologists can induce mutations using different methods, which in most cases are not desirable and precise. But, now molecular biologists can alter any amino acid of a protein by changing the corresponding bases in its gene very precisely and accurately resulting in desirable mutations. It is possible to alter properties such as increased stability, temperature resistance, product inhibition, substrate specificity, etc. of any enzyme.

The accurate induction of one or more point mutations on selected regions of a gene resulting in amino acid substitutions or deletion or addition is known as **sitedirected mutagenesis**. It can be defined as the controlled alteration of selected regions of a DNA molecule.

The **principle of site-directed mutagenesis** is that a mismatched oligonucleotide primer is extended, incorporating the 'mutation' into a strand of DNA that can be cloned. This technique of creating desired molecular mutations in a gene has contributed greatly to the basic understanding of functions of genes, DNA-protein interactions, gene regulations, the role of amino acids in the structure and functions of proteins, role of active centres in the enzyme-substrate interactions, etc. A single base change in a gene permits the evaluation of the role of specific amino acids in the function and structure of a protein. This technique also allows one to create or destroy a restriction site at specific locations within a DNA sequence or gene.

Site-directed mutagenesis is actually one of the applications of PCR. The gene, which has to be mutated, should be made into a single-stranded DNA by cloning into a M13 plasmid vector. By following modern PCR methods, it is possible to carry out the site directed mutation without the participation of M13 vector. The designing and chemical synthesis of the primer is the key factor in this technique. The part of the DNA where the mutation has to be introduced should be synthesized as an oligonucleotide primer, which is complementary to the respective region of the DNA except for the nucleotide that has to be changed. In short, the mutation is introduced to the gene in the form of a primer and the primer is extended with a polymerase reaction.

The site-directed mutagenesis is a multistep process that begins with the cloning of the gene in a bacteriophage like M13 to generate single stranded DNA. M13 is a filamentous bacteriophage that specifically infects *e. coli* that expresses sex pili encoded by a plasmid F factor. M13 bacteriophage contains DNA in a single-stranded or replicative form, which is replicated to double-stranded DNA within a bacterial cell. The primer is designed and synthesized, which is an oligonucleotide complementary to the region of the DNA to be mutated except for the nucleotide to be changed. This oligonucleotide with the mismatched base or bases hybridize to the single-stranded DNA and serve as the primer to start synthesizing the complementary DNA strand with the help of a suitable DNA polymerase such as T4 DNA polymerase or Taq polymerase. The resulting doublestranded DNA will be a hybrid of the wild type parent strand and the mutated newly synthesized DNA strand. This DNA molecule can be transformed into an E.*coli* cell, where the mutated DNA strand serves as a template to replicate new strands carrying the mutation along with the wild strands. The bacteriophage plaques containing the mutated DNA can be screened by hybridizing with the labeled probe of the original mutated oligonucleotide. By adjusting the hybridization time and wash temperature of the hybridized probe, only the perfectly matched hybrid will remain and all other mismatched hybrids will dissociate. The presence of the desired mutation in the gene can be checked and confirmed after isolating the plasmid DNA from the single positive plaques and sequencing it.

A single base can be mutated in recombinant DNA plasmids with a process called **inverse PCR.** Two primers are synthesized with their antiparallel 5' ends complementary to the adjacent bases on the two strands of DNA. One of the two primers carries a specific mismatched base that is faithfully copied during the PCR amplification resulting in a recombinant plasmid with a single mutated base.

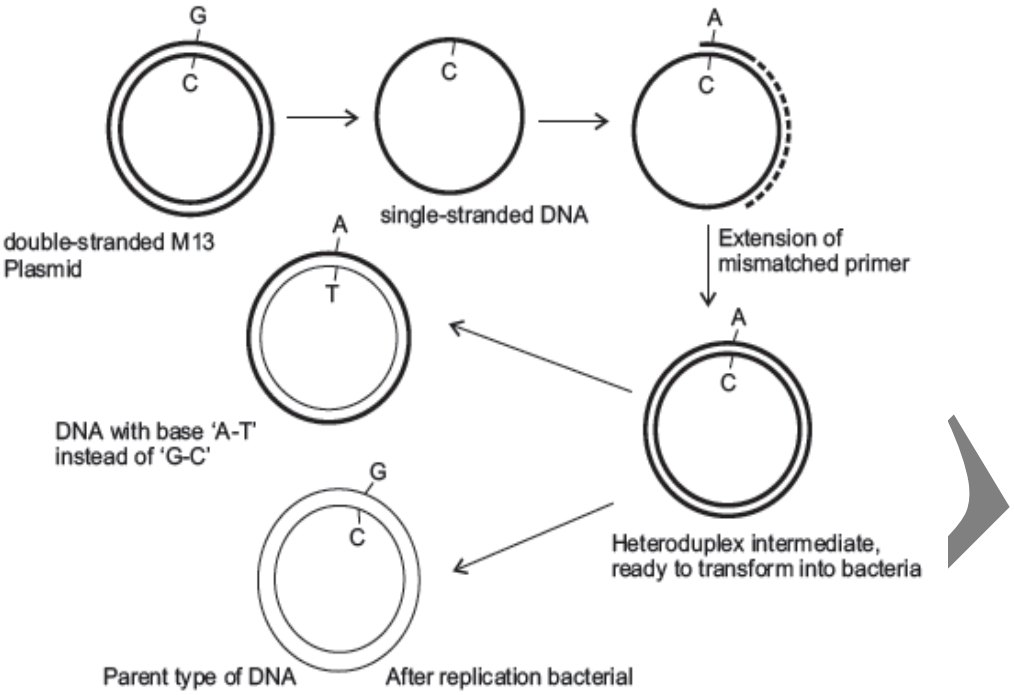


FIGURE: Site-directed mutagenesis of a gene mediated through M13 plasmid.

DNA micro array

A DNA microarray (also commonly known as **gene** or **genome** chip, DNA chip, or gene array) is a collection of microscopic DNA spots, commonly representing single genes, arrayed on a solid surface by covalent attachment to chemically suitable matrices.

DNA arrays are different from other types of microarray, only in that they either measure DNA or use DNA as part of its detection system.

Qualitative or quantitative measurements with DNA microarrays utilize the selective nature of DNA-DNA or DNA-RNA hybridization under high-stringency conditions and fluorophore-based detection. DNA arrays are commonly used for expression profiling, i.e., monitoring expression levels of thousands of genes simultaneously, or for comparative genomic hybridization.

Arrays of DNA can either be spatially arranged, as in commonly known gene or genome chip, DNA chip, or gene array, or can be specific DNA sequences tagged or labelled such that they can be independently identified in solution. The traditional solid-phase array is a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic or silicon chip.

The affixed DNA segments are known as probes (although some sources will use different nomenclature such as reporters), thousands of which can be placed in known locations on a single DNA microarray. Microarray technology evolved from Southern blotting, whereby fragmented DNA is attached to a substrate and then probed with a known gene or fragment.

Fabrication:

Microarrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto glass slides, photolithography using pre-made masks, photolithography using dynamic micro-mirror devices, ink-jet printing, or electrochemistry on microelectrode arrays.

DNA microarrays can be used to detect RNAs that may or may not be translated into active proteins. Scientists refer to this kind of analysis as "expression analysis" or expression profiling. Since there can be tens of thousands of distinct probes on an array, each microarray experiment can accomplish the equivalent number of genetic tests in parallel.

Arrays have, therefore, dramatically accelerated many types of investigations. The use of microarrays for gene expression profiling was first published in 1995 (Science) and the first complete eukaryotic genome {Saccharomyces cerevisiae) on a microarray was published in 1997 (Science).

1. Spotted Microarrays:

In spotted microarrays (or two-channel or two-colour microarrays), the probes are oligonucleotides, cDNA or small fragments of PCR products that correspond to mRNAs and are spotted onto the microarray surface. This type of array is typically hybridized with cDNA from two samples to be compared (e.g., diseased tissue versus healthy tissue) that are labelled with two different fluorophores (e.g., Rhodamine (Cyanine 5, red) and Fluorescein (Cyanine 3, green)).

The two samples are mixed and hybridized to a single microarray that is then scanned in a microarray scanner to visualize fluorescence of the two fluorophores. Relative intensities of each fluorophore are then used to identify up-regulated and down-regulated genes in ratio-based analysis. Absolute levels of gene expression cannot be determined in the two-colour array, but relative differences in expression among different spots (= genes) can be estimated with some oligonucleotide arrays.

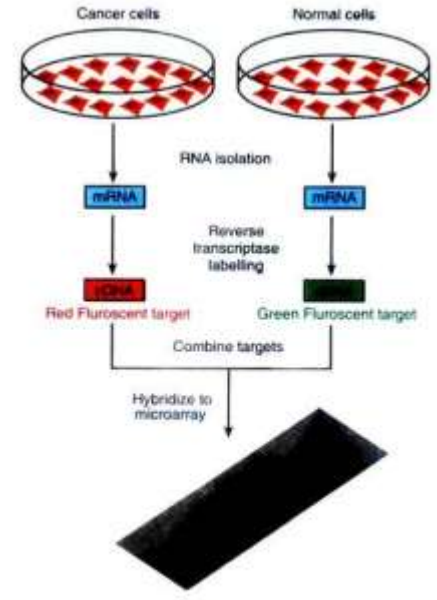


Fig. 16.2: Diagram of typical dual-colour microarray experiment

2. Oligonucleotide Microarrays:

In oligonucleotide microarrays (or single-channel microarrays), the probes are designed to match parts of the sequence of known or predicted mRNAs. There are commercially available designs that cover complete genomes from companies such as GE Healthcare, Affymetrix, Ocimum Bio-solutions, or Agilent. These microarrays give estimations of the absolute value of gene expression and, therefore, the comparison of two conditions requires the use of two separate micro- arrays.

Fig. 16.3: Two Affymetrix chips

Oligonucleotide Arrays can be either produced by piezoelectric deposition with full length oligonucleotides or in situ synthesis. Long Oligonucleotide Arrays are composed of 60-mers, or 50-mers and are produced by ink-jet printing on a silica substrate. Short Oligonucleotide Arrays are composed of 25-mer or 30-mer and are produced by photolithographic synthesis (Affymetrix) on a silica substrate or piezoelectric deposition (GE Healthcare) on an acrylamide matrix.

More recently, Maskless Array Synthesis from NimbleGen Systems has combined flexibility with large numbers of probes. Arrays can contain up to 390,000 spots, from a custom array design. New array formats are being developed to study specific pathways or disease states for a systems biology approach.

Oligonucleotide microarrays often contain control probes designed to hybridize with RNA spike-ins. The degree of hybridization between the spike-ins and the control probes is used to normalize the hybridization measurements for the target probes.

Genotyping Microarrays:

DNA microarrays can also be used to read the sequence of a genome in particular positions. SNP microarrays are a particular type of DNA microarrays that are used to identify genetic variation in individuals and across populations.

Short oligonucleotide arrays can be used to identify the single nucleotide polymorphisms (SNPs) that are thought to be responsible for genetic variation and the source of susceptibility to genetically caused diseases. Generally termed genotyping applications, DNA microarrays may

be used in this fashion for forensic applications, rapidly discovering or measuring genetic predisposition to disease, or identifying DNA-based drug candidates.

These SNP microarrays are also being used to profile somatic mutations in cancer, specifically loss of heterozygosity events and amplifications and deletions of regions of DNA. Amplifications and deletions can also be detected using comparative genomic hybridization, or aCGH, in conjunction with microarrays, but may be limited in detecting novel Copy Number Polymorphisms, or CNPs, by probe coverage.

Re-sequencing arrays have also been developed to sequence portions of the genome in individuals. These arrays may be used to evaluate germ line mutations in individuals, or somatic mutations in cancers. Genome tiling arrays include overlapping oligonucleotides designed to blanket an entire genomic region of interest. Many companies have successfully designed tiling arrays that cover whole human chromosomes.

Microarrays and Bioinformatics:

1. Experimental Design:

Due to the biological complexity of gene expression, the considerations of experimental design that are discussed in the expression profiling article are of critical importance if statistically and biologically valid conclusions are to be drawn from the data.

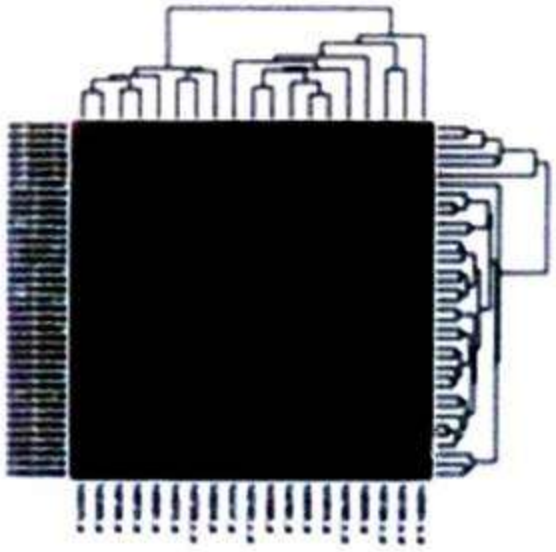


Fig. 16.4: Gene expression values from microarray experiments can be represented as heat maps to visualize the result of data analysis

There are three main elements to consider when designing a microarray experiment.

First, replication of the biological samples is essential for drawing conclusions from the experiment.

Second, technical replicates (two RNA samples obtained from each experimental unit) help to ensure precision and allow for testing differences within treatment groups. The technical replicates may be two independent RNA extractions or two aliquots of the same extraction.
Third, spots of each cDNA clone or oligonucleotide are present at least as duplicates on the microarray slide, to provide a measure of technical precision in each hybridization. It is critical that information about the sample preparation and handling is discussed in order to help identify the independent units in the experiment as well as to avoid inflated estimates of significance.

2. Standardization:

The lack of standardization in arrays presents an interoperability problem in bioinformatics, which hinders the exchange of array data. Various grass-roots open-source projects are attempting to facilitate the exchange and analysis of data produced with non-proprietary chips.
1. The "Minimum Information about a Microarray Experiment" (MIAME) checklist helps define the level of detail that should exist and is being adopted by many journals as a requirement for the submission of papers incorporating microarray results. MIAME describes the minimum required information for complying experiments, but not its format. Thus, as of 2007, whilst many formats can support the MIAME requirements there is no format which permits verification of complete semantic compliance.
2. The "MicroArray Quality Control (MAQC) Project" is being conducted by the FDA to develop standards and quality control metrics which will eventually allow the use of MicroArray data in drug discovery, clinical practice and regulatory decision-making.
3. The MicroArray and Gene Expression (MAGE) group is working on the standardization of the representation of gene expression data and relevant annotations.

3. Statistical Analysis:

The analysis of DNA microarrays poses a large number of statistical problems, including the normalization of the data. There are dozens of proposed normalization methods in the published literature; as in many other cases where authorities disagree, a sound conservative approach is to try a number of popular normalization methods and compare the conclusions reached; how sensitive are the main conclusions to the method chosen?
From a hypothesis-testing perspective, the large number of genes present on a single array means that the experimenter must take into account a multiple testing problem; even if the statistical P-value assigned to a given gene indicates that it is extremely unlikely that differential expression of this gene was due to random rather than treatment effects, the very high number of genes on an array makes it likely that differential expression of some genes represents false positives or false negatives.
Statistical methods tailored to microarray analyses have recently become available that assess statistical power based on the variation present in the data and the number of experimental replicates, and can help minimize type I and type II errors in the analyses.
A basic difference between microarray data analysis and much traditional biomedical research is the dimensionality of the data. A large clinical study might collect 100 data items per patient for thousands of patients. A medium-size microarray study will obtain many thousands of numbers per sample for perhaps a hundred samples. Many analysis techniques treat each sample as a single point in a space with thousands of dimensions, then attempt by various techniques to reduce the dimensionality of the data to something humans can visualize.

4. Relation between Probe and Gene:

The relation between a probe and the mRNA that it is expected to detect is problematic. On the one hand, some mRNAs may cross-hybridize probes in the array that are supposed to detect another mRNA. On the other hand, probes that are designed to detect the mRNA of a particular gene may be relying on genomic EST information that is incorrectly associated with that gene.

Public Databases of Microarray Data:

Database	Microarray Experiment Sets	Sample Profiles	As of Date
Gene Expression Omnibus - NCBI	5366	134669	April 1, 2007
Stanford Microarray database	12742	?	April 1, 2007
UNC Microarray database	~31	2093	April 1, 2007
MUSC database	~45	555	April 1, 2007
ArrayExpress at EBI	1643	136	April 1, 2007
caArray at NCI	41	1741	November 15, 2006

Online Microarray Data Analysis Programs and Tools:

Several Open Directory Project categories list online microarray data analysis programs and tools:

i. Bioinformatics: Online Services:

Gene Expression and Regulation at the Open Directory Project

ii. Gene Expression:

Databases at the Open Directory Project

iii. Gene Expression:

Software at the Open Directory Project

iv. Data Mining:

Tool Vendors at the Open Directory Project

v. Bio-conductor:

Open source and open development software project for the analysis and comprehension of genomic data

vi. Genevestigator:

Web-based database and analysis tool to study gene expression across large sets of tissues, developmental stages, drugs, stimuli, and genetic modifications.

Applications of these Arrays include:

1. mRNA or gene expression profiling:

Monitoring expression levels for thousands of genes simultaneously is relevant to many areas of biology and medicine, such as studying treatments, disease, and developmental stages. For example, microarrays can be used to identify disease genes by comparing gene expression in diseased and normal cells.

2. Comparative genomic hybridization (Array CGH):

Assessing large genomic rearrangements.

3. SNP detection arrays:

Looking for single nucleotide polymorphism in the genome of populations.

4. Chromatin immunoprecipitation (ChIP) studies:
Determining protein binding site occupancy throughout the genome, employing ChlP-on-chip technology.

POSSIBLE QUESTIONS

8 MARKS

1. Give a detailed account on PCR techniques and its applications.
2. Write a detailed note on site directed mutagenesis
3. Explain the method of DNA sequencing in detail.
4. Discuss in detail about DNA microarray and its applications.
5. Discuss the topic DNA foot and finger printing in detail.
6. What are hybridization techniques? How it is employed in molecular biology?
7. Give a detailed account on hybridization probes.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
III-B.Sc., BIOCHEMISTRY
15BCU603A –CORE ELECTIVE II-INTRODUCTION TO BIOTECHNOLOGY
MULTIPLE CHOICE QUESTIONS

UNIT-IV

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	_____ genes are encoded on the Ti plasmid of *A. tumefaciens*	*nif* genes	*ras* genes	*vir* genes	*coz*	***vir* genes**
2	_____ permits the plasmid to be stably maintained in *A. tumefaciens*	Ti plasmid	Ori region	Vir genes	Opine catabolism region	**Ori region**
3	Tms1 or aux1 in T-DNA encodes	isopentyl transferase	indole 3- acetamide hydralase	Tryptophan –2-monoxygenase	None	**Tryptophan –2-monoxygenase**
4	Tms2 or aux2 in T-DNA encodes	isopentyl transferase,	indole 3- acetamide hydralase	Tryptophan –2-monoxygenase	None	**indole 3-acetamide hydralase**
5	Fructopine synthesis is encoded by	mas gene	ags gene	frs gene	tmr	**frs gene**
6	In T-DNA region, the gene which encodes isopentenyl transferease is	*tmr*	*tms1*	*tms2*	*nif*	***tmr***
7	Crown gall tumors are induced by	*E. coli.*	*Agrobacterium tumefaciens*	*Pseudomonas aeruginosa*	*Bacillus sp.*	***Agrobacterium tumefaciens***
8	Tms1 or aux1 in T-DNA encodes	isopentyl transferase	indole 3- acetamide hydralase	Tryptophan –2-monoxygenase	None	**Tryptophan –2-monoxygenase**
9	The length of T-DNA region can vary from	12 to 24 kb	10 to 20 kb	14 to 26 kb	9 to 19 kb	**12 to 24 kb**
10	_____, a phenolic compound, is responsible for the activation of vir genes of *A. tumefaciens* .	Acetosyringone	Acetylcholine	ADP	ATP	**Acetosyringone**
11	The plasmid found in virulent strain of A.	Ti plasmid	Pla	Ri plasmid	S	**Ri plasmid**
12	In ti plasmid the operon required for virulence is	VIR B	VIR C	VIR H	VIR F	**VIR B**
13	_____ is a major group of oncogenic Ti plasmid	Histipine	Octopinic acid	Lysopine	Octopine	**Octopine**
14	The genes that provide endonucleases to initiate the transfer process by nicking T-DNA at a specific process are	vir C1 and vir C2	vir D, vir D1 and vir D2	vir H	vir E2	**vir D, vir D1 and vir D2**
15	The proteins that contain a nuclear targeting sequence to direct the T- strand to the nucleus of the transformed cells are	vir D1	vir E2	vir H	vir D2	**vir D2**
16	Mannopine synthesis is encoded by	*ma* s gene	*ags* gene	*frs* gene	*tm* r	***ma* s gene**
17	*ags* gene encodes	Octapine synthesis	agropine synthesis	Nopaline synthesis	mannopine synthesis	**agropine synthesis**
18	Octopine synthesis is encoded by	Nos gene	Ocs gene	Frs gene	Ags gene	**Ocs gene**
19	Nopaline synthase is encoded by	Nos gene	Ocs gene	Frs gene	Ags gene	**Ocs gene**

20	Hairy root disease in higher is caused by	*tumefaciens*	*rhizogenes*	*E.coli*	*Bacillus* sp.	***rhizogenes***
21	_____ has been used to transfer DNA via pollen as a vector to overcome the nuclease action on DNA	*E.coli*	*P. aeruginosa*	*Bacillus* sp	*Tumefaciens*	***Tumefaciens***
22	The delivery of viral viroidal sequence to plants using bacterium as route is	Direct delivery system	Vector less transfer	Agro infection	Electroporation	**Electroporation**
23	Cauliflower mosaic virus (CaMV) is potential vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	None of the above	**caulimoviruses**
24	Dahlia mosaic virus is a vector cited under the group	caulimoviruses	Gemini viruses	RNA viruses	None of the above	**caulimoviruses**
25	In CaMV, _____ of open reading frames codes the insect transmission	ORF I	ORF II	Minor ORF	None of the above	**ORF II**
26	_____ is the process where electrical impulses of high strength size used for DNA transfer	Microprojectiles	Particle bombardment	Electroporation	Biolistics	**Electroporation**
27	_____ used to assist the association of the DNA with membrane in Electroporation mediated DNA transfer	Polyethylene glycol	Gun powder	Silicon-Carbide	Calcium	**Polyethylene glycol**
28	A metal used in particle bombardment DNA transfer is	Thorium	Silver	Tungsten	Iron	**Tungsten**
29	The gas used in microprojectile bombardment is	Argon	Helium	Neon	Nitrogen	**Helium**
30	_____ DNA transfer has the advantage of avoiding protoplast isolation	Liposome mediated transformation	Silicon carbide fiber transformation	Ultrasound mediated transformation	DNA transfer via pollen	**Silicon carbide fiber transformation**
31	The genes with overdrive sequence to stimulates the T-DNA transfer process are	vir C1 and vir C2	vir D, vir D1 and vir D2	vir H	vir E2	**vir C1 and vir C2**
32	Mitochondial DNA are ----------	circular and double stranded	circular and single stranded	doule helical	single linear	**circular and double stranded**
33	_____ operon is essential for virulence in Agrobacterium transformation.	vir A	vir E2	vir H	vir B	**vir B**
34	_____ gene with ATP binding site involved in delivering energy required for T-DNA transfer	vir B11	vir E2	vir H	vir A	**vir B11**
35	The genes present in _____ operon have a role in the detoxification of certain plant compounds that might affect the growth of Agrobacterium	vir A	vir E2	vir H	vir B	**vir H**
36	Maize streak virus vector is a member of	RNA viruses	Caulimoviruses	Gemini viruses	None of the above	**Gemini viruses**
37	The DNA of gemini virus is	Double stranded	Coiled	Single stranded	Both single and double	**Single stranded**
38	TMV and bromo mosaic viruses are the members of	caulimoviruses	RNA viruses	Gemini viruses	None of the options	**RNA viruses**

39	_____ is the monopartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	**TMV**
40	_____ is the multipartite RNA virus	TMV	Tobacco ringspot virus	BMV	Tomato black ring virus satellite	**BMV**
41	---------- is one of the most wide spread commercial GM trait	Herbicide resistance	Glyphosate resistance	EPSPS gene	Phosphinothricin	**Glyphosate resistance**
42	Glyphosate is effective against	Grasses	Trees	Crops	None	**Grasses**
43	Phosphinothricin is effective against	Grasses	Trees	Crops	Broad leaved weeds	**Broad leaved weeds**
44	Bacillus thuringiensis was discovered by	Ishiwaki	Maagdki	Boulter	Crick	**Ishiwaki**
45	---------- is an example of defense response gene	chitinase	Cellulose	ribozyme	Catalase	**chitinase**
46	_____ genes are encoded on the Ti plasmid of *A. tumefaciens*	Genes	*ras* genes	*vir* genes	*coz*	***vir* genes**
47	_____ permits the plasmid to be stably maintained in *A. tumefaciens*	Ti plasmid	Ori region	Vir genes	Opine catabolism region	**Ori region**
48	Nopaline synthase is encoded by	Nos gene	Ocs gene	Frs gene	Ags gene	**Ocs gene**
49	_____ is the process where electrical impulses of high strength size used for DNA transfer	Microprojectiles	Particle bombardment	Electroporation	Biolistics	**Electroporation**
50	In CaMV, _____ of open reading frames codes the insect transmission	ORF I	ORF II	Minor ORF	None of the above	**ORF II**
51	_____ RNAs are unable to self replicate in the infected plants	Satellite RNAs	Monopartite viruses	Multipartite viruses	Subgenomic RNAs	**Subgenomic RNAs**
52	The first plant RNA viral vector is	RNA IV virus	BMV	Tobacco ringspot virus satellite	TMV vector TB2	**TMV vector TB2**
53	In TMV, the foreign gene expression and ORF of coat protein is derived by	subgenomic promoter of BMV	subgenomic promoter of tobacco ringspot virus	odontoglossum ringspot tobamovirus subgenomic promoter	Spg of tomato black ring virus satellite	**odontoglossum ringspot tobamovirus subgenomic promoter**
54	----------is a bacterium that parasitizes the caterpillars of some harmful ---moths and butterflies.	*Bacillus thuringiensis*	*E. coli.*	*Agrobacterium tumefaciens*	*Pseudomonas aeruginosa*	***Bacillus thuringiensis***
55	Environmentally safe ways to attack pests such as the gypsy moth, the tent caterpillar, and the tobacco hornworm is	Spraying plants with spores of *Bacillus thuringiensis*	Dusting plants with spores of *Bacillus thuringiensis*	Both a and b	etching spores of *Bacillus thuringiensis*	**Both a and b**
56	Defense response genes include	Hydrolytic enzyme	RIPs	AFPs	All the above	**All the above**
57	Which of the following is a organic pesticide	Bovistin	Bt crystals	Azospirilllum	Cyanobacteria	**Bt crystals**
58	Which Bt protein is used in potato crops to produce resistance against beetle	Cry3A	Cry1A	Cry9C	cry1Ab	**Cry3A**
59	---------gene in tomato confers resistance to strains of pseudomonas	pto gene	RPW gene	fen gene	xa21 gene	**pto gene**

60	The property of pr protein tobacco pr 1a is	bind protease	plant defensing	antifungal	endochitinase	**antifungal**

UNIT-IV
SYLLABUS

Gene transfer techniques- Microinjection, biolistic methods, vector based transfer.Plant tissue culture-Media composition, nutrients and growth regulators, callus culture.Genetic engineering of plants-methodology- plant transformation with Ti plasmidof Agrobacterium tumifaciens. Production of herbicide resistance plant (with reference to glyphosate only). Applications of transgenic plants.

Gene transfer techniques

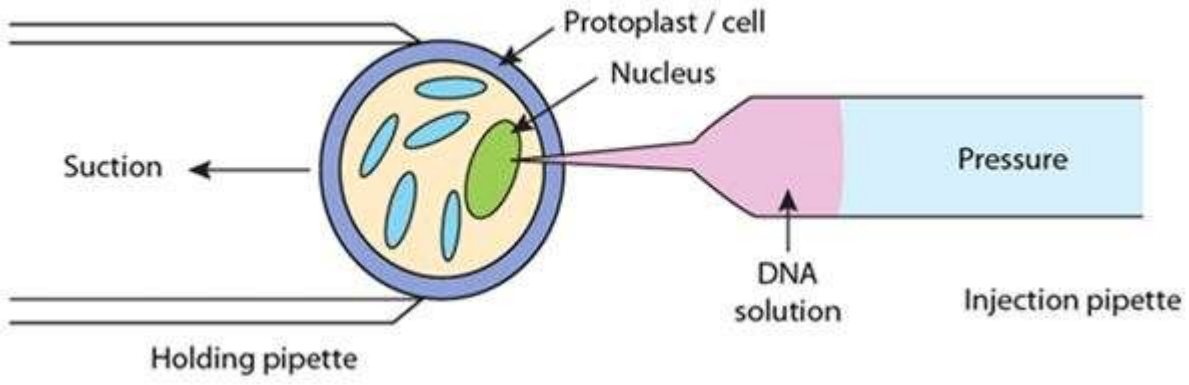
Microinjection

The process of using a fine glass micropipette to manually inject transgene at microscopic or borderline macroscopic level is known as microinjection.The transgene, in the form of plasmids, cosmids, phage, YACs, or PCR products, can be circular or linear and need not be physically linked for injection.

Microinjection involves direct mechanical introduction of DNA intothe nucleus or cytoplasm using a glass microcapillary injectionpipette. The protoplasts are immobilized in low melting agar, while working under a microscope, using a holding pipette and suction force. DNA is then directly injected into the cytoplasm or the nucleus. Theinjected cells are then cultured *invitro* and regenerated into plants. Successful examples of this process has been shown in rapeseed, tobacco and various other plants.

Stable transformants can be achieved through this method but it requires technical expertise and is atime consuming process. Also,microinjection has achieved only limited success in plant transformationdue to the thick cell walls of plants and a lack of availability of a single-cell-to-plant regenerationsystem in most plant species.

In this technique a traditional compound microscope (around 200X magnification) or aninverted microscope (around 200x magnification) or a dissecting stereomicroscope (around 40-50x)is used.Under the microscope target cell is positioned and cell membrane and nuclear envelope are penetrated with the help of two micromanipulators.One micromanipulator holds the pipette and another holds the microcapillary needle.



There are two types of microinjection systems; constant flow system and pulsed flow system.

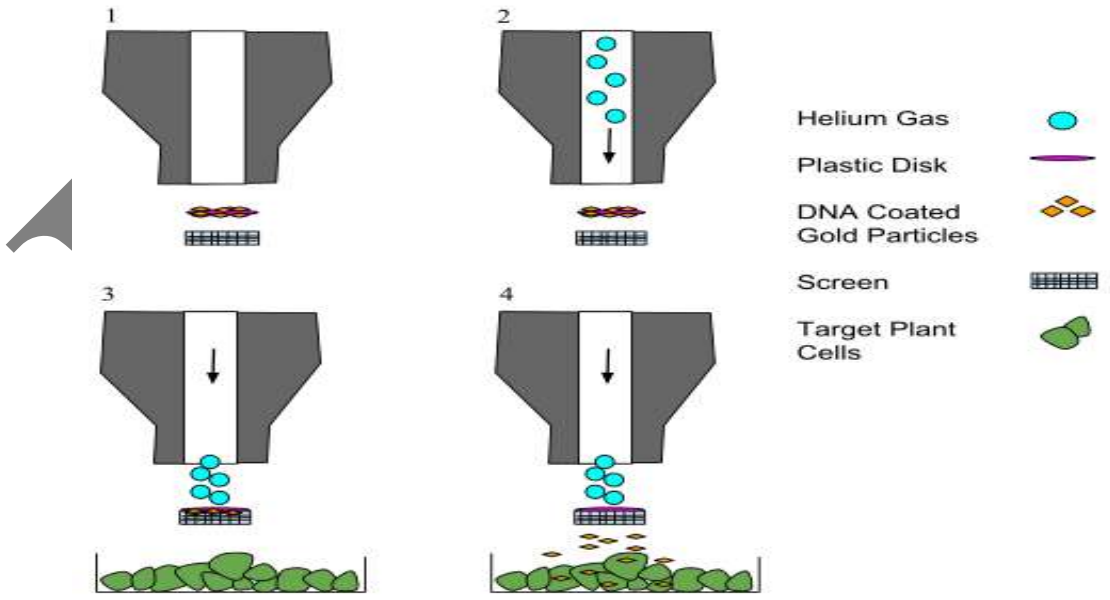
- In the constant flow system the amount of sample injected is determined by the duration for which needle remains in the cell. The constant flow system is relatively simple and inexpensive but outdated.
- The pulsed flow system has greater control over the volume of substance delivered, needle placement and movement and has better precision. This technique results in less damage to the receiving cell, however, the components of this system are quite expensive.

Biolistic methods

A gene gun or a biolistic particle delivery system, originally designed for plant transformation, is a device for delivering exogenous DNA (transgenes) to cells. The payload is an elemental particle of a heavy metal coated with DNA (typically plasmid DNA). This technique is often simply referred to as biolistics.

This device is able to transform almost any type of cell, including plants, and is not limited to transformation of the nucleus; it can also transform organelles, including plastids.

A gene gun is used for delivery of exogenous DNA to cells. This method is known as 'biolistics'. Gene guns can be used effectively on most cells but are mainly used on plant cells. Step 1 The gene gun apparatus is ready to fire. Step 2 Helium fills the chamber and pressure builds against the rupture disk. Step 3 The pressure eventually reaches the point where the rupture disk breaks, and the resulting burst of helium propels the DNA/gold-coated macrocarrier ('Plastic Disk') into the stopping screen. Step 4 When the macrocarrier hits the stopping screen, the DNA-coated gold particles are propelled through the screen and into the target cells.



Advantages

Biolistics has proven to be a versatile method of genetic modification and it is generally preferred to engineer transformation-resistant crops, such as cereals. Notably, Bt maize is a

product of biolistics. Plastid transformation has also seen great success with particle bombardment when compared to other current techniques, such as Agrobacterium mediated transformation, which have difficulty targeting the vector to and stably expressing in the chloroplast. In addition, there are no reports of a chloroplast silencing a transgene inserted with a gene gun. Additionally, with only one firing of a gene gun, a skilled technician can generate two transformed organisms. This technology has even allowed for modification of specific tissues in situ, although this is likely to damage large numbers of cells and transform only some, rather than all, cells of the tissue.

Vector based transfer

The salient features of the commonly used gene (DNA) transfer methods are given in Table 49.1.

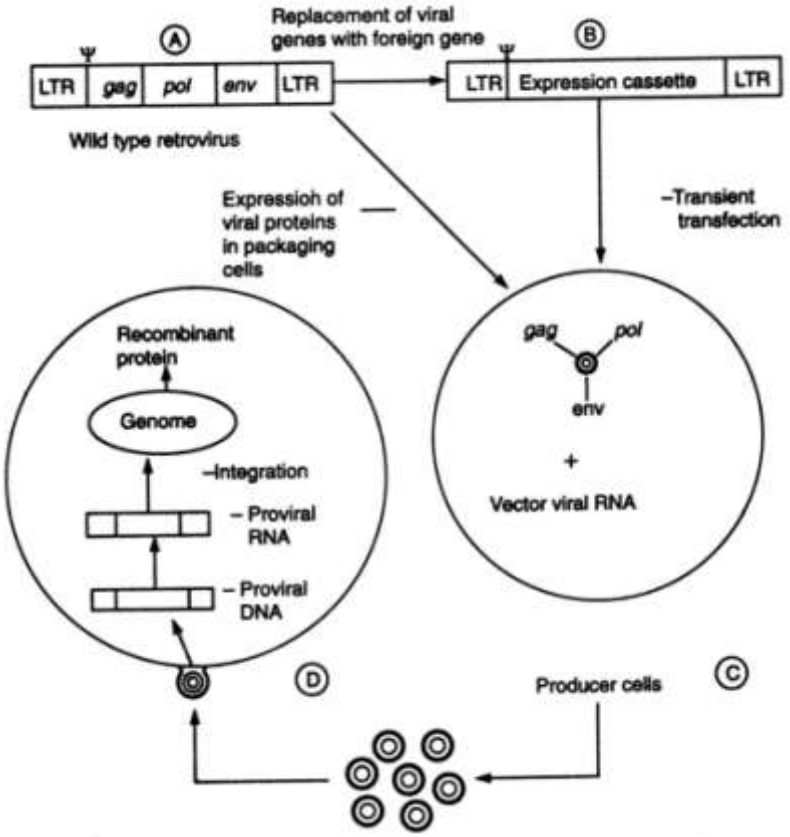
TABLE 49.1 Gene transfer (DNA delivery) methods in plants

Method	*Salient features*
I. Vector–mediated gene transfer	
Agrobacterium (Ti plasmid)-mediated gene transfer	Very efficient, but limited to a selected group of plants
Plant viral vectors	Ineffective method, hence not widely used
II. Direct or vectorless DNA transfer	
(A) Physical methods	
Electroporation	Mostly confined to protoplasts that can be regenerated to viable plants. Many cereal crops developed.
Microprojectile (particle bombardment)	Very successful method used for a wide range of plants/ tissues. Risk of gene rearrangement high.
Microinjection	Limited use since only one cell can be microinjected at a time. Technical personnel should be highly skilled.
Liposome fusion	Confined to protoplasts that can be regenerated into viable whole plants.
Silicon carbide fibres	Requires regenerable cell suspensions. The fibres, however, require careful handling.
(B) Chemical methods	
Polyethylene glycol (PEG)-mediated	Confined to protoplasts. Regeneration of fertile plants is frequently problematical.
Diethylaminoethyl (DEAE) dextran-mediated	Does not result in stable transformants.

A variety of gene transfer strategies have been developed during the last decade for the treatment of human diseases which can be grouped into the two major categories: the viral and non-viral methods.

Virus vectors: After 1980, much work has been done on retroviruses as gene transfer vectors, more specifically on murine-leukemia virus (MLV) for gene therapy. Efforts are being made to develop HIV-based vectors so that even non-dividing cells can be injected. The steps of developing a replication-defective recombinant retroviral vectors are : *(i)* the replacement of viral structural genes *e.g. gag, pol* and *env* by the therapeutic foreign genes of interest, *(ii)* transfection of this vector into packaging cell line *(i.e.* producer cells) that provide the viral structural proteins *in trans* so that the recombinant retroviral genome is packed and replication defective

retroviruses are generated, *(iii)* transfection of host cells by such viruses, and reverse transcription of recombinant retroviral RNA and random integration into the host genome. In the absence of viral genes, the foreign genes (therapeutic in nature is transcribed from the viral LTRs, the long terminal repeats) and desired protein is synthesized. The retroviral vectors are used in *ex vivo* gene transfer experiment, although it has been shown that they can infect a regenerating liver when administered intravenously into hepatectomized animal.



Plant tissue culture - Media composition, nutrients and growth regulators

Plant tissue culture is the technique of maintaining and growing plant cells, tissues or organs especially on artificial medium in suitable containers under controlled environmental conditions. The part which is cultured is called explant, i.e., any part of a plant taken out and grown in a test tube, under sterile conditions in special nutrient media. This capacity to generate a whole plant from any cell/explant is called cellular toti-potency. In fact, the whole plant can be regenerated from any plant part (referred to as explant) or cells. Gottlieb Haberlandt first initiated tissue culture technique in 1902.

Hormones used in Plant Tissue Culture:

1. Auxins neoline (Indole-3-acetic acid, Indole-3-butyric acid, Potassium Salt— Naphthalene acetic acid 2, 4-Dichlorophenoxyacetic acid p-Chloro-phenoxy acetic acid)
2. Cytokinins (6-Benzylaminopurine, 6-Dimethylallylaminopurine (2ip), Kinetin)
3. Gibberellins (Gibberellic Acid)

4. Abscisic Acid (ABA) (Abscisic Acid)
5. Polyamines (Putrescine, Spermidine)

Environmental Conditions:
There are three important aspects *in vitro*
(i) nutrient medium,
(ii) aseptic conditions and
(iii) aeration of the tissue

1. Nutrient Medium:
The composition of plant tissue culture medium can vary depending upon the type of plant tissues or cell that are used for culture. A typical (generalized) nutrient consists of inorganic salts (both micro and macro elements), a carbon source (usually sucrose), vitamins (e.g., nicotonic acid, thiamine, pyridoxine and myoinositol), amino acids (e.g., arginine) and growth regulators (e.g., auxins like 2,4-D or 2,4-dichlorophenoxyacetic acid and cytokinins such as BAP = benzlaminopurine and gibberellins). Other compounds like casein hydrolysate, coconut milk, malt extract, yeast extract, tomato juice, etc. may be added for specific purposes.
Plant hormones play important role in growth and differentiation of cultured cells and tissues. An optimum pH (usually 5.7) is also very important. The most extensively used nutrient medium is MS medium which was developed by Murashige and Skoog in 1962. Usually a gelling agent agar (a polysaccharide obtained from a red algae Gelidium amansi) is added to the liquid medium for its solidification.

2. Aseptic Conditions (Sterilization):
Nutrient medium contains ample sugar which increases growth of microorganisms such as bacteria and fungi. These microbes compete with growing tissue and finally kill it. It is essential to maintain aseptic conditions of tissue culture. Thus sterilization means complete destruction or killing of microorganisms so that complete aseptic conditions are created for in vitro culturing.

3. Aeration of the Tissue:
Proper aeration of the cultured tissue is also an important aspect of culture technique. It is achieved by occasionally stirring the medium by sterring or by automatic shaker.

Plant Material—the Explant:
Any part of a plant taken out and grown in test tube under sterile conditions in special nutrient media is called explant.

Methods of Plant Tissue Culture:
Plant tissue culture includes two major methods:
(A) Type of in vitro growth-callus and suspension cultures.
(B) Type of explant— single cell culture, shoot and root cultures, somatic embryo culture, meristem culture, anther culture and haploid production, protoplast culture and somatic hybridisation, embryo culture, ovule culture, ovary culture, etc.

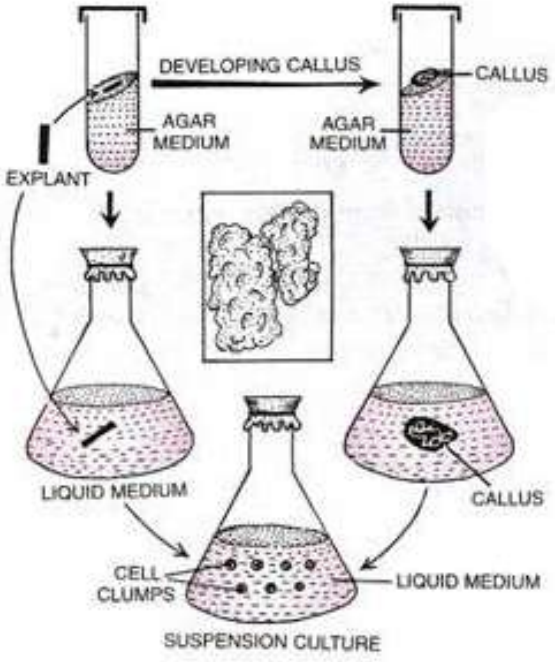
Callus Cultures
In callus culture, cell division in explant forms a callus. Callus is irregular unorganised and undifferentiated mass of actively dividing cells. Darkness and solid medium gelled by agar stimulates callus formation. The medium ordinarily contains the auxin, 2,4-D, (2, 4-

dichlorophenoxy acetic acid) and often a cytokinin like BAP (Benzyl aminopurine). Both are growth regulators. This stimulates cell divison in explant. Callus is obtained within 2-3 weeks.

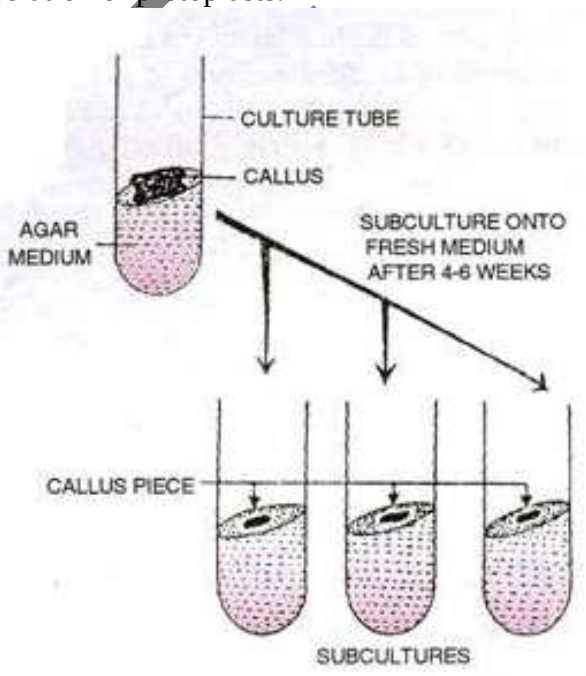
Sub culturing:

If tissue cultures are kept in the same culture vessel, they die in due course of time. Therefore, cells/tissues are regularly transferred into new culture vessels containing fresh media. This process is called sub culturing. It is important to note that during subculture; only a part of the culture from a vessel is transferred into the new culture vessel.

The callus and suspension cultures may be used to achieve cell biomass production, regeneration of plantlets, production of transgenic plants and isolation of protoplasts.



Initiation of callus and suspension cultures.



Schematic representation of subculturing.

Micro propagation:

Micropropagation is the tissue culture technique used for rapid vegetative multiplication of ornamental plants and fruit trees by using small sized explants. Because of minute size of the propagules in the culture, the propagation technique is named as mircopropagation. This method of tissue culture produces several plants. Each of these plants will be genetically identical to the original plant from where they were grown.

The genetically identical plants developed from any part of a plant by tissue culture/micropropagation are called somaclones. The members of a single somaclone have the same genotype. This micropropagation is also known as somaclonal propagation. It is the only process adopted by Indian plant biotechnologists in different industries mainly for the commercial production of ornamental plants like lily, orchids, Euca lyptus, Cinchona, Blueberry, etc. and fruit trees like tomato, apple, banana, grapes, potato, citrus oil palm, etc.

There are four defined steps in micro propagation method. These are:
(i) Initiation of culture from an explant like shoot tip on a suitable nutrient medium.
(ii) Shoot formation multiple shoots formation from the cultured explant.
(iii) Rooting of shoots rooting of in vitro developed shoots.
(iv) Transplantation the hardening of tissue culture raised plants and subsequent transplantation to the field.

Advantages of Micro propagation:

These are as follows:
1. It helps in rapid multiplication of plants.
2. A large number of plantlets are obtained within a short period and from a small space.
3. Plants are obtained throughout the year under controlled conditions, independent of seasons.
4. Sterile plants or plants which cannot maintain their characters by sexual reproduction are multiplied by this method.
5. It is an easy, safe and economical method for plant propagation.
6. In case of ornamentals, tissue culture plants give better growth, more flowers and less fall-out.
7. Genetically similar plants (somaclones) are formed by this method. Therefore, desirable characters (genetope) and desired sex of superior variety are kept constant for many generations.
8. The rare plant and endangered species are multiplied by this method and such plants are saved.

Regeneration of Plantlets:

1. Preparation of Suitable Nutrient Medium:

Suitable nutrient medium as per objective of culture is prepared and transferred into suitable containers.

2. Selection of Explants:

Selection of explants such as shoot tip should be done.

3. Sterilisation of Explants:

Surface sterilization of the explants by disinfectants and then washing the explants with sterile distilled water is essential.

4. Inoculation:

Inoculation (transfer) of the explants into the suitable nutrient medium (which is sterilized by filter-sterilized to avoid microbial contamination) in culture vessels under sterile conditions is done.

5. Incubation:

Growing the culture in the growth chamber or plant tissue culture room, having the appropriate physical condition (i.e., artificial light; 16 hours of photoperiod), temperature (-26°C) and relative humidity (50-60%) is required.

6. Regeneration:

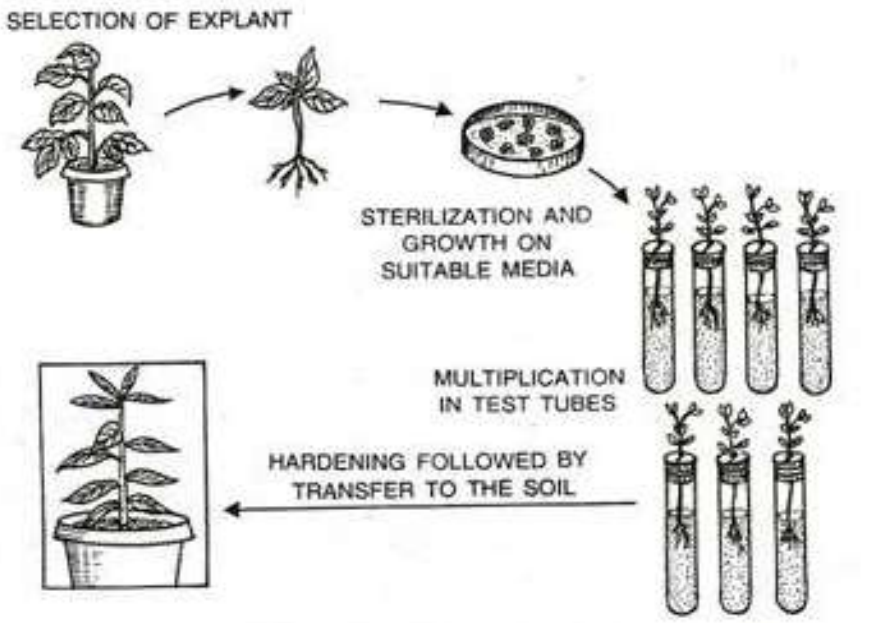
Regeneration of plants from cultured plant tissues is carried out.

7. Hardening:

Hardening is gradual exposure of plantlets to an environmental conditions.

8. Plantlet Transfer:

After hardening plantlets transferred to the green house or field conditions following acclimatization (hardening) of regenerated plants.



Regeneration of whole plants using tissue culture technique.

Practical Applications of Plant Tissue Culture:

The use of plant cells to generate useful products and/or services constitutes plant biotechnology. In plant biotechnology, the useful product is a plantlet. The plantlets are used for the following purposes.

1. Rapid Clonal Propagation:

A clone is a group of individuals or cells derived from a single parent individual or cell through asexual reproduction. All the cells in callus or suspension culture are derived from a single explant by mitotic division. Therefore, all plantlets regenerated from a callus/suspension culture generally have the same genotype and constitute a clone. These plantlets are used for rapid clonal propagation. This is done in oil palm.

2. Somaclonal Variation:

Genetic variation present among plant cells of a culture is called somaclonal variation. The term somaclonal variation is also used for the genetic variation present in plants regenerated from a single culture. This variation has been used to develop several useful varieties.

3. Transgenic Plants:

A gene that is transferred into an organism by genetic engineering is known as transgene. An organism that contains and expresses a transgene is called transgenic organism. The transgenes can be introduced into individual plant cells. The plantlets can be regenerated from these cells. These plantlets give rise to the highly valuable transgenic plants.

4. Induction and Selection of Mutations:

Mutagens are added to single cell liquid cultures for induction of mutations. The cells are washed and transferred to solid culture for raising mutant plants. Useful mutants are selected for further

breeding. Tolerance to stress like pollutants, toxins, salts, drought, flooding, etc. can also be obtained by providing them in culture medium in increasing dosage. The surviving healthy cells are taken to solid medium for raising resistant plants.

5. Resistance to Weedicides:

It is similar to induction of mutations. Weedicides are added to culture initially in very small concentrations. Dosage is increased in subsequent cultures till the desired level of resistance is obtained. The resistant cells are then regenerated to form plantlets and plants.

Genetic engineering of plants

Genetically modified crops (GMCs, GM crops, or biotech crops) are plants used in agriculture, the DNA of which has been modified using genetic engineering techniques. In most cases, the aim is to introduce a new trait to the plant which does not occur naturally in the species.

Organization of Ti plasmid:

The Ti plasmids (approximate size 200 kb each) exist as independent replicating circular DNA molecules within the Agrobacterium cells. The T-DNA (transferred DNA) is variable in length in the range of 12 to 24 kb, which depends on the bacterial strain from which Ti plasmids come. Nopaline strains of Ti plasmid have one T-DNA with length of 20 kb while octopine strains have two T-DNA regions referred to as T_L and T_R that are respectively 14 kb and 7 kb in length.

A diagrammatic representation of a Ti plasmid is depicted in Fig. The Ti plasmid has three important regions.

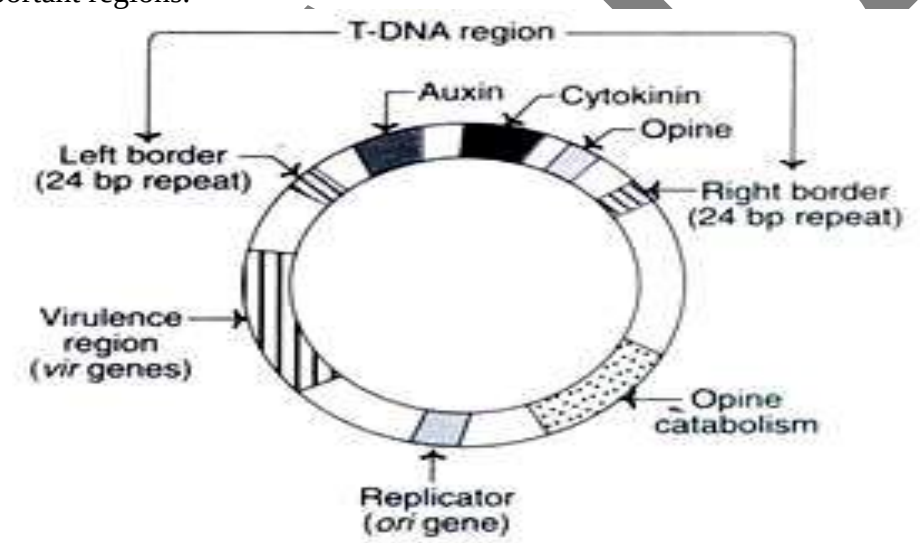


Fig. 49.3 : A dagrammatic representation of a Ti plasmid.

1. T-DNA region:

This region has the genes for the biosynthesis of auxin (aux), cytokinin (cyt) and opine (ocs), and is flanked by left and right borders. These three genes-aux, cyto and ocs are referred to as oncogenes, as they are the determinants of the tumor phenotype.

T-DNA borders — A set of 24 kb sequences present on either side (right and left) of T-DNA are also transferred to the plant cells. It is now clearly established that the right border is more critical for T-DNA transfer and tumori-genesis.

2. Virulence region:

The genes responsible for the transfer of T-DNA into the host plant are located outside T-DNA and the region is referred to as vir or virulence region. Vir region codes for proteins involved in T-DNA transfer. At least nine vir-gene operons have been identified. These include vir A, vir G, vir B_1, vir C_1, vir D_1, D_2 and D_4, and vir E_1, and E_2.

3. Opine catabolism region:

This region codes for proteins involved in the uptake and metabolisms of opines. Besides the above three, there is ori region that is responsible for the origin of DNA replication which permits the Ti plasmid to be stably maintained in A. tumefaciens.

T-DNA transfer and integration:

The process of T-DNA transfer and it integration into the host plant genome is depicted in Fig. 49.4, and is briefly described.

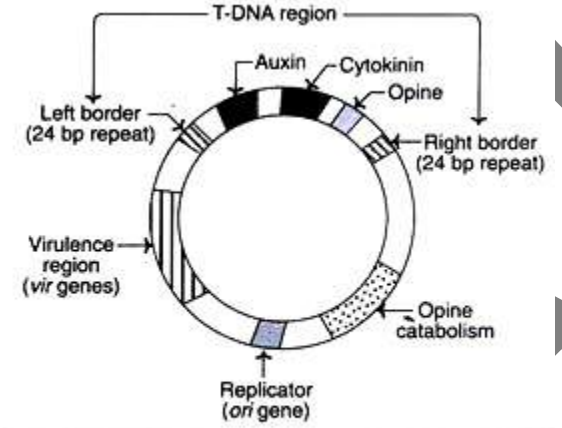


Fig. 49.3 : A dagrammatic representation of a Ti plasmid.

1. Signal induction to Agrobacterium:

The wounded plant cells release certain chemicals- phenolic compounds and sugars which are recognized as signals by Agrobacterium. The signals induced result in a sequence of biochemical events in Agrobacterium that ultimately helps in the transfer of T-DNA of T-plasmid.

2. Attachment of Agrobacterium to plant cells:

The Agrobacterium attaches to plant cells through polysaccharides, particularly cellulose fibres produced by the bacterium. Several chromosomal virulence (chv) genes responsible for the attachment of bacterial cells to plant cells have been identified.

3. Production of virulence proteins:

As the signal induction occurs in the Agrobacterium cells attached to plant cells, a series of events take place that result in the production of virulence proteins. To start with, signal induction by phenolics stimulates vir A which in turn activates (by phosphorylation) vir C. This induces expression of virulence genes of Ti plasmid to produce the corresponding virulence

proteins (D1, D2, E_2, B etc.). Certain sugars (e.g. glucose, galactose, xylose) that induce virulence genes have been identified.

4. Production of T-DNA strand:

The right and left borders of T-DNA are recognized by vir D_1/vir D_2proteins. These proteins are involved in the production single-stranded T-DNA (ss DNA), its protection and export to plant cells. The ss T-DNA gets attached to vir D_2.

5. Transfer of T-DNA out of Agrobacterium:

The ss T-DNA — vir D_2 complex in association with vir G is exported from the bacterial cell. Vir B products form the transport apparatus.

6. Transfer of T-DNA into plant cells and integration:

The T-DNA-vir D_2 complex crosses the plant plasma membrane. In the plant cells, T-DNA gets covered with vir E_2. This covering protects the T-DNA from degradation by nucleases; vir D_2 and vir E_2 interact with a variety of plant proteins which influences T-DNA transport and integration.

The T-DNA-vir D_2-vir E_2 — plant protein complex enters the nucleus through nuclear pore complex. Within the nucleus, the T-DNA gets integrated into the plant chromosome through a process referred to illegitimate recombination. This is different from the homologous recombination, as it does not depend on the sequence similarity.

Hairy Root Disease of A. Rhizogenes — R_1 Plasmids:

Agrobacterium rhizogenes can also infect plants. But this results in hairy roots and not crown galls as is the case with A. tumefaciens. The plasmids, of A. rhizogenes have been isolated and characterized. These plasmids, referred to as Ri plasmids, (Ri stands for Root inducing) are of different types. Some of the Ri plasmid strains possess genes that are homologous to Ti plasmid e.g. auxin biosynthetic genes.

Instead of virulence genes, Ri plasmids contain a series of open reading frames on the T-DNA. The products of these genes are involved in the metabolism of plant growth regulators which gets sensitized to auxin and leads to root formation.

Vectors of A. rhizogenes:

As it is done with A tumefaciens, vectors can be constructed by using A. rhizogenes. These vectors are alternate strategies for gene transfer. However, employment of A. rhizogene-based vectors for plant transformation is not common since more efficient systems of A. tumefaciens have been developed.

Importance of hairy roots:

Hairy roots can be cultured in vitro, and thus are important in plant biotechnology. Hairy root systems are useful for the production of secondary metabolites, particularly pharmaceutical proteins.

Ti Plasmid-Derived Vector Systems:

The ability of Ti plasmid of Agrobacterium to genetically transform plants has been described. It is possible to insert a desired DNA sequence (gene) into the T-DNA region (of Ti plasmid), and then use A. tumefaciens to deliver this gene(s) into the genome of plant cell.

In this process, Ti plasmids serve as natural vectors. However, there are several limitations to use Ti plasmids directly as cloning vectors:

i. Ti plasmids are large in size (200-800 kb). Smaller vectors are preferred for recombinant experiments. For this reason, large segments of DNA of Ti plasmid, not essential for cloning, must be removed.
ii. Absence of unique restriction enzyme sites on Ti plasmids.
iii. The phytohormones (auxin, cytokinin) produced prevent the plant cells being regenerated into plants. Therefore auxin and cytokinin genes must be removed.
iv. Opine production in transformed plant cells lowers the plant yield. Therefore opine synthesizing genes which are of no use to plants should be removed.
v. Ti plasmids cannot replicate in E. coli. This limits their utility as E. coli is widely used in recombinant experiments. An alternate arrangement is to add an origin of replication to Ti plasmid that allows the plasmid to replicate in E. coli.
Considering the above limitations, Ti plasmid- based vectors with suitable modifications have been constructed.

These vectors are mainly composed of the following components:

1. The right border sequence of T-DNA which is absolutely required for T-DNA integration into plant cell DNA.
2. A multiple cloning site (poly-linker DNA) that promotes the insertion of cloned gene into the region between T-DNA borders.
3. An origin of DNA replication that allows the plasmids to multiply in E. coli.
4. A selectable marker gene (e.g. neomycin phosphotransferase) for appropriate selection of the transformed cells.

Two types of Ti plasmid-derived vectors are used for genetic transformation of plants— co-integrate vectors and binary vectors.

Co-integrate vector:

In the co-integrate vector system, the disarmed and modified Ti plasmid combines with an intermediate cloning vector to produce a recombinant Ti plasmid (Fig. 49.5).

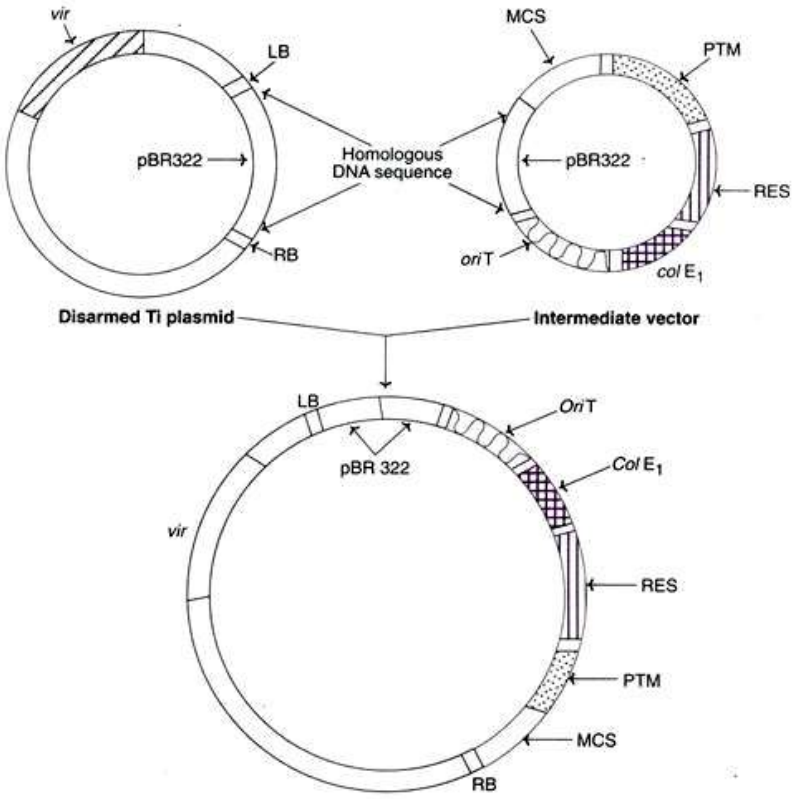


Fig. 49.5 : Cointegrate vector system (vir-Ti plasmid virulence region; pBR322-Bacterial plasmid 322; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; col E_1-Origin of a replication from col E_1 plasmid; ori T-Origin of transfer site for conjugative plasmid mobilization).

Production of disarmed Ti plasmid:

The T-DNA genes for hormone biosynthesis are removed (disarmed). In place of the deleted DNA, a bacterial plasmid (pBR322) DNA sequence is incorporated. This disarmed plasmid, also referred to as receptor plasmid, has the basic structure of T-DNA (right and left borders, virulence genes etc.) necessary to transfer the plant cells.

Construction of intermediate vector:

The intermediate vector is constructed with the following components:

i. A pBR322 sequence DNA homologous to that found in the receptor Ti plasmid.

ii. A plant transformation marker (PTM) e.g. a gene coding for neomycin phosphotransferase II (npt II). This gene confers resistance to kanamycin in the plant cells and thus permits their isolation.

iii. A bacterial resistance marker e.g. a gene coding for spectinomycin resistance. This gene confers spectinomycin resistance to recipient bacterial cells and thus permits their selective isolation.

iv. A multiple cloning site (MCS) where foreign genes can be inserted.

v. A Co/E_1 origin of replication which allows the replication of plasmid in E. coli but not in Agrobacterium.
vi. An oriT sequence with basis of mobilization (bom) site for the transfer of intermediate vector from E. coli to Agrobacterium.

Production and use of co-integrate vectors:

The desired foreign gene (target-gene) is first cloned in the multiple cloning site of the intermediate vector. The cloning process is carried out in E. coli, the bacterium where the cloning is most efficient. The intermediate vector is mated with Agrobacterium so that the foreign gene is mobilised into the latter.

The transformed Agrobacterium cells with receptor Ti plasmid and intermediate vector are selectively isolated when grown on a minimal medium containing spectinomycin. The selection process becomes easy since E. coli does not grow on a minimal medium in which Agrobacterium grows.

Within the Agrobacterium cells, intermediate plasmid gets integrated into the receptor Ti plasmid to produce co-integrate plasmid. This plasmid containing plant transformation marker (e.g. npt II) gene and cloned target gene between T-DNA borders is transferred to plant cells. The transformed plant cells can be selected on a medium containing kanamycin when the plant and Agrobacterium cells are incubated together.

Advantages of co-integrate vector:

i. Target genes can be easily cloned
ii. The plasmid is relatively small with a number of restriction sites.
iii. Intermediate plasmid is conveniently cloned in E. coli and transferred to Agrobacterium.

Binary vector:

The binary vector system consists of an Agrobacterium strain along with a disarmed Ti plasmid called vir helper plasmid (the entire T-DNA region including borders deleted while vir gene is retained). It may be noted that both of them are not physically linked (or integrated). A binary vector with T-DNA can replicate in E. coli and Agrobacterium.

A diagrammatic representation of a typical binary vector system is depicted in Fig. 49.6. The binary vector has the following components.

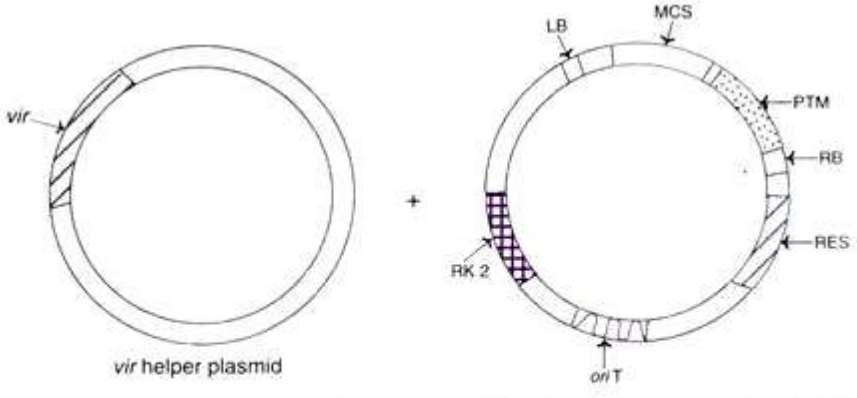


Fig. 49.6 : Binary vector system (vir-Ti plasmid virulence region; LB-Left border; RB-Right border; MCS-Multiple cloning site; PTM-Plant transformation marker; RES-Bacterial resistance marker; oriT-Origin of transfer site for conjugative plasmid mobilization; RK_2-Origin of replication from plasmid).

1. Left and right borders that delimit the T-DNA region.
2. A plant transformation marker (PTM) e.g. npt II that confers kanamycin resistance in plant transformed cells.
3. A multiple cloning site (MCS) for introducing target/foreign genes.
4. A bacterial resistance marker e.g. tetracycline resistance gene for selecting binary vector colonies in E. coli and Agrobacterium.
5. oriT sequence for conjugal mobilization of the binary vector from E. coli to Agrobacterium.
6. A broad host-range origin of replication such as RK_2 that allows the replication of binary vector in Agrobacterium.

Production and use of binary vector:

The target (foreign) gene of interest is inserted into the multiple cloning site of the binary vector. In this way, the- target gene is placed between the right and left border repeats and cloned in E. coli. By a mating process, the binary vector is mobilised from E. coli to Agrobacterium. Now, the virulence gene proteins of T-DNA facilitate the transfer of T-DNA of the vector into plant cells.

Advantages of binary vectors:

i. The binary vector system involves only the transfer of a binary plasmid to Agrobacterium without any integration. This is in contrast to co-integrate vector system wherein the intermediate vector is transferred and integrated with disarmed Ti plasmid.
ii. Due to convenience, binary vectors are more frequently used than co-integrate vectors.

Plant Transformation Technique Using Agrobacterium

Agrobacterium-mediated technique is the most widely used for the transformation of plants and generation of transgenic plants. The important requirements for gene transfer in higher plants through Agrobacterium mediation are listed.
i. The explants of the plant must produce phenolic compounds (e.g. autosyringone) for activation of virulence genes.
ii. Transformed cells/tissues should be capable to regenerate into whole plants.
In general, most of the Agrobacterium-mediated plant transformations have the following basic protocol (Fig. 49.7)

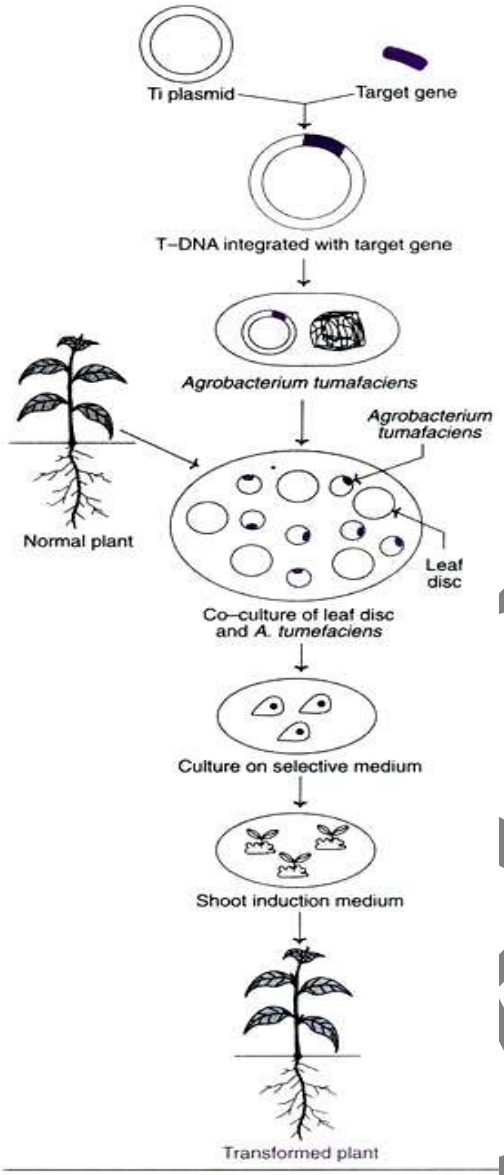


Fig. 49.7 : Transformation technique using Agrobacterium-mediated gene transfer.

1. Development of Agrobacterium carrying the co-integrate or binary vector with the desired gene.
2. Identification of a suitable explant e.g. cells, protoplasts, tissues, calluses, organs.
3. Co-culture of explants with Agrobacterium.
4. Killing of Agrobacterium with a suitable antibiotic without harming the plant tissue.
5. Selection of transformed plant cells.
6. Regeneration of whole plants.

Advantages of Agrobacterium- mediated transformation:

i. This is a natural method of gene transfer.
ii. Agrobacterium can conveniently infect any explant (cells/tissues/organs).

iii. Even large fragments of DNA can be efficiently transferred.
iv. Stability of transferred DNA is reasonably good.
v. Transformed plants can be regenerated effectively.

Limitations of Agrobacterium- mediated transformation:

i. There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g. cereals) are not infected by it. In recent years, virulent strains of Agrobacterium that can infect a wide range of plants have been developed.
ii. The cells that regenerate more efficiently are often difficult to transform, e.g. embryonic cells lie in deep layers which are not easy targets for Agrobacterium.

Herbicide Tolerance Technology

Weeds are constant problem in farmers' fields. Weeds not only compete with crops for water, nutrients, sunlight, and space but also harbor insect and disease pests; clog irrigation and drainage systems; undermine crop quality; and deposit weed seeds into crop harvests. If left uncontrolled, weeds can reduce crop yields significantly.

Farmers can fight weeds with tillage, hand weeding, herbicides, or typically a combination of all techniques. Unfortunately, tillage leaves valuable topsoil exposed to wind and water erosion, a serious long-term consequence for the environment. For this reason, more and more farmers prefer reduced or no-till methods of farming.

Similarly, many have argued that the heavy use of herbicides has led to groundwater contaminations, the death of several wildlife species and has also been attributed to various human and animal illnesses.

Advantages of Herbicide Tolerant Crops

- Excellent weed control and hence higher crop yields;
- Flexibility – possible to control weeds later in the plant's growth
- Reduced numbers of sprays in a season;
- Reduced fuel use (because of less spraying);
- Reduced soil compaction (because of less need to go on the land to spray);
- Use of low toxicity compounds which do not remain active in the soil; and
- The ability to use no-till or conservation-till systems, with consequent benefits to soil structure and organisms

Herbicide resistance plant

Weeds (wild herbs) are unwanted and useless plants that grow along with the crop plants. Weeds compete with traps for light and nutrients, besides harbouring various pathogens. It is estimated that the world's crop yield is reduced by 10-15% due to the presence of weeds.

To tackle the problem of weeds, modern agriculture has developed a wide range of weed killers which are collectively referred to as herbicides. In general, majority of the herbicides are broad-spectrum as they can kill a wide range of weeds.

A good or an ideal herbicide is expected to possess the following characteristics:

i. Capable of killing weeds without affecting crop plants.
ii. Not toxic to animals and microorganisms.
iii. Rapidly trans-located within the target plant.
iv. Rapidly degraded in the soil.

None of the commercially available herbicides fulfills all the above criteria. The major limitation of the herbicides is that they cannot discriminate weeds from crop plants. For this reason, the crops are also affected by herbicides, hence the need to develop herbicide-resistant plants. Thus, these plants provide an opportunity to effectively kill the weeds (by herbicides) without damaging the crop plants.

Strategies for engineering herbicide resistance:

A number of biological manipulations particularly involving genetic engineering are in use to develop herbicide-resistant plants.

1. Overexpression of the target protein:

The target protein, being acted by the herbicide can be produced in large quantities so that the affect of the herbicide becomes insignificant. Overexpression can be achieved by integrating multiple copies of the genes and/or by using a strong promoter.

2. Improved plant detoxification:

The plants do possess natural defense systems against toxic compounds (herbicides). Detoxification involves the conversion of toxic herbicide to non-toxic or less toxic compound. By enhancing the plant detoxification system, the impact of the herbicide can be reduced.

3. Detoxification of herbicide by using a foreign gene:

By introducing a foreign gene into the crop plant, the herbicide can be effectively detoxified.

4. Mutation of the target protein:

The target protein which is being affected by the herbicide can be suitably modified. The changed protein should be capable of discharging the functions of the native protein but is resistant to inhibition by the herbicide.

Once the resistant target protein gene is identified, it can be introduced into the plant genomes, and thus herbicide-resistant plants can be developed. For success in the development of herbicide resistant plants, good knowledge of the target protein and the action of herbicides is required.

Some of the developments made in the herbicide resistance of plant are briefly described:

Glyphosate Resistance:

Glyphosate, is a glycine derivative. It acts as a broad-spectrum herbicide and is effective against 76 of the world's worst 78 weeds. Glyphosate is less toxic to animals and is rapidly degraded by microorganisms. In addition, it has a short half-life. The American chemical company Monsanto markets glyphosate as Round up.

Mechanism of action of glyphosate:

Glyphosate is rapidly transported to the growing points of plants. It is capable of killing the plants even at a low concentration. Glyphosate acts as a competitive inhibitor of the enzyme 5-enoylpyruvylshikimate 3-phosphate synthase (EPSPS). This is a key enzyme in shikimic acid pathway that results in the formation of aromatic amino acids (tryptophan, phenylalanine and tyrosine), phenols and certain secondary metabolites (Fig. 50.5).

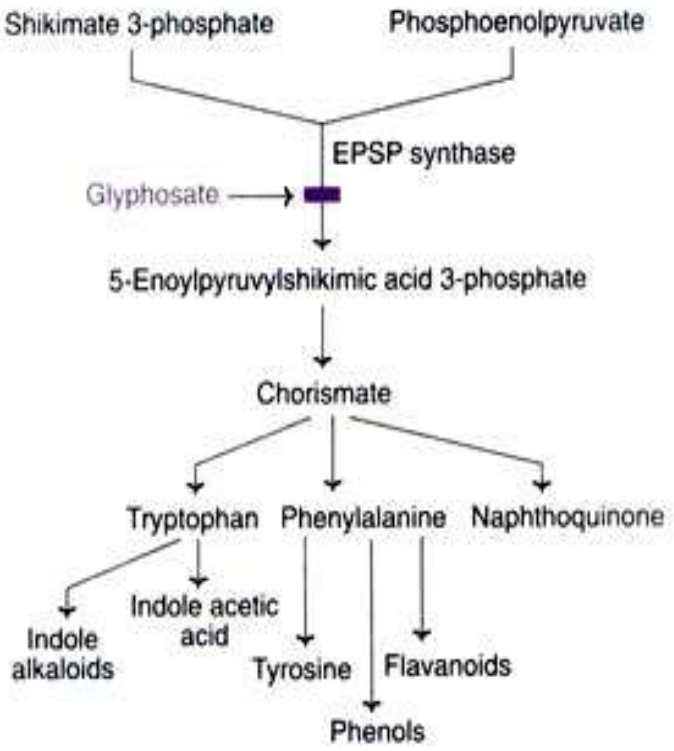
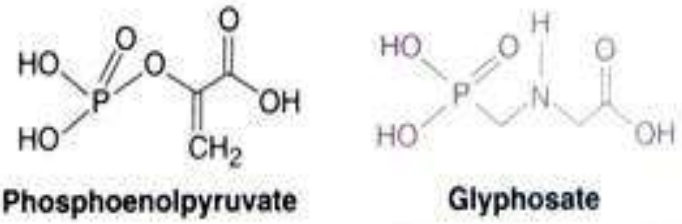


Fig. 50.5 : Shikimate pathway indicating the action of the herbicide glyphosate (EPSP synthase-5-Enolylpyruvylshikimate 3-phosphate synthase)

The enzyme EPSPS catalyses the synthesis of 5-enoylpyruvylshikimate 3-phosphate from shikimate 3-phosphate and phosphoenoylpyruvate. Glyphosate has some structural similarly with the substrate phosphoenol pyruvate (Fig 50.6). Consequently, glyphosate binds more tightly with EPSPS and blocks the normal shikimic acid pathway. Thus, the herbicide glyphosate inhibits the biosynthesis of aromatic amino acids and other important products.



Phosphoenolpyruvate **Glyphosate**

Fig. 50.6 : Structures of phosphoenolpyruvate (the substrate) and the herbicide glyphosate (the competitive inhibitor).

This results in inhibition of protein biosynthesis (due to lack of aromatic amino acids). As a consequence, cell division and plant growth are blocked. Further, the plant growth regulator indole acetic acid (an auxin) is also produced from tryptophan. The net result of glyphosate is the death of the plants. Glyphosate is toxic to microorganisms as they also possess shikimate pathway.

Glyphosate is non-toxic to animals (including humans), since they do not possess shikimate pathway. Of the three aromatic amino acids (synthesized in this pathway), tryptophan and phenylalanine are essential and they have to be supplied in the diet, while tyrosine can be formed from phenylalanine.

Strategies for glyphosate resistance:

There are three distinct strategies to provide glyphosphate resistance to plants:

1. Overexpression of crop plant EPSPS gene:

An overexpressing gene of EPSPS was detected in Petunia. This expression was found to be due to gene amplification rather than an increased expression of the gene. EPSPS gene from Petunia was isolated and introduced into other plants. The increased synthesis of EPSPS (by about 40 fold) in transgenic plants provides resistance to glyphosate. These plants can tolerate glyphosate at a dose of 2-4 times higher than that required to kill wild-type plants.

2. Use of mutant EPSPS genes:

An EPSPS mutant gene that conferred resistance to glyphosate was first detected in the bacterium Salmonella typhimurium. It was found that a single base substitution (C to 7) resulted in the change of an amino acid from proline to serine in EPSPS. This modified enzyme cannot bind to glyphosate, and thus provides resistance.

The mutant EPSPS gene was introduced into tobacco plants using Agrobacterium Ti plasmid vectors. The transgene produced high quantities of the enzyme EPSPS. However, the transformed tobacco plants provided only marginal resistance to glyphosate. The reason for this was not immediately identified.

It was later known that the shikimate pathway occurs in the chloroplasts while the glyphosate resistant EPSPS was produced only in the cytoplasm. This enzyme was not transported to the chloroplasts, hence the problem to provide resistance. This episode made scientists to realize the importance of chloroplasts in genetic engineering.

In later years, the mutant EPSPS gene was tagged with a chloroplast-specific transit peptide sequence. By this approach, the glyphosate-resistant EPSPS enzyme was directed to freely enter chloroplast and confer resistance against the herbicide.

3. Detoxification of glyphosate:

The soil microorganisms possess the enzyme glyphosate oxidase that converts glyphosate to glyoxylate and aminomethylphosponic acid. The gene encoding glyphosate oxidase has been isolated from a soil organism Ochrobactrum anthropi. With suitable modifications, this gene was introduced into crop plants e.g. oilseed rape. The transgenic plants were found to exhibit very good glyphosate resistance in the field.

Use of a combined strategy:

More efficient resistance of plants against glyphosate can be provided by employing a combined strategy. Thus, resistant (i.e. mutant) EPSPS gene in combination with glyphosate oxidase gene

are used. By this approach, there occurs glyphosate resistance (due to mutant EPSPS gene) as well as its detoxification (due to glyphosate oxidase gene).

Resistance to other herbicides:

Besides the above, some other herbicide resistant plants have also been developed e.g. bromoxynil, atrazine, phenocarboxylic acids, cyanamide. A list of selected examples of gene transferred herbicide resistant plants is given in Table 50.7.

TABLE 50.7 Selected examples of gene transferred herbicide resistant plants

Herbicide	*Gene transfer/mechanism of resistance*	*Transgenic crop(s)*
Glyphosate	Inhibition of EPSPS	Soybean, tomato
Glyphosate	Detoxification by glyphosate oxidase	Maize, soybean
Phosphinothricin	*bar* gene coding phosphinothricin acetyltransferase	Maize, rice, wheat, cotton, potato, tomato, sugarbeet
Sulfonylureas imidazolinones	Mutant plant with acetolactate synthase	Rice, tomato, maize, sugarbeet
Bromoxynil	Nitrilase detoxification	Cotton, potato, tomato
Atrazine	Mutant plant with chloroplast *psb A* gene	Soybean
Phenocarboxylic acids	Monooxygenase detoxification (e.g. 2,4-D and 2,4,5-T)	Maize cotton
Cyanamide	Cyanamide hydratase gene	Tobacco

It may however, be noted that some of the herbicide-resistant transgenic plants are at field-trial stage. Due to environmental concern, a few of these plants are withdrawn e.g. atrazine- resistant crops.

Environmental Impact of Herbicide-Resistant Crops:

The development genetically modified (GM) herbicide-resistant crops has undoubtedly contributed to increase in the yield of crops. For this reason, farmers particularly in the developed countries (e.g. USA) have started using these GM crops. Thus, the proportion of herbicide resistant soybean plants grown in USA increased from 17% in 1997 to 68% in 2001.

The farmer is immensely benefited as there is a reduction in the cost of herbicide usage. It is believed that the impact of herbicide-resistant plants on the environment is much lower than the direct use of the herbicides in huge quantities.

There are however, other environmental concerns:

i. Disturbance in biodiversity due to elimination of weeds.

ii. Rapid development of herbicide-resistance weeds that may finally lead to the production of super weeds.

Goals of biotechnological improvements in crops:

There are about 30-40 crops that have been genetically modified, and many more are being added. However, very few of them have got the clearance for commercial use. A selected list is already given in Table 50.9.

The ultimate goals of genetically modified (CM) crop plants are listed below:

i. Resistance to diseases (insect, microorganisms).

ii. Improved nitrogen fixing ability.

iii. Higher yielding capacity.
iv. Resistance to drought and soil salinity.
v. Better nutritional properties.
vi. Improved storage qualities.
vii. Production of pharmaceutically important compounds.
viii. Absence of allergens.
ix. Modified sensory attributes e.g. increased sweetness as in thaumatin.

Concerns about transgenic plants:

The fears about the harmful environmental and hazardous health effects of transgenic plants still exist, despite the fact that there have been no reports so far in this regard. The transfer of almost all the transgenic plants from the laboratory to the crop fields is invariably associated with legal and regulatory hurdles, besides the social and economic concerns.

The major concern expressed by public (also acknowledged by biotechnologists) is the development of resistance genes in insects, generation of super weeds etc. Several remedial measures are advocated to overcome these problems.

The farmers in developing countries are much worried about the seed terminator technology which forces them to buy seeds for every new crop. These farmers are traditionally habituated to use the seeds from the previous crop which is now not possible due to seed terminator technology.

6. Transgenic Plants as Bioreactors:

Another important application of genetically transformed plants is their utility as bioreactors to produce a wide range of metabolic and industrial products.

Applications of Transgenic Plants

The genetic manipulations carried out in plants for the production of transgenic plants have been described .The ultimate goal of transgenic (involving introduction, integration, and expression of foreign genes) is to improve the crops, with the desired traits.

Some of the important ones are listed:

i. Resistance to biotic stresses i.e. resistance to diseases caused by insects, viruses, fungi and bacteria.
ii. Resistance to abiotic stresses-herbicides, temperature (heat, chilling, freezing), drought, salinity, ozone, intense light.
iii. Improvement of crop yield, and quality e.g. storage, longer shelf life of fruits and flowers.
iv. Transgenic plants with improved nutrition.
v. Transgenic plants as bioreactors for the manufacture of commercial products e.g. proteins, vaccines, and biodegradable plastics.

Environmental stresses to plants:

The different types of external stresses that influence the plant growth and development are depicted in Fig. 50.1, These stresses are grouped based on their characters—biotic and abiotic stresses. The biotic stresses are caused by insects, pathogens (viruses, fungi, bacteria), and wounds. The abiotic stresses are due to herbicides, water deficiency (caused by drought, temperature, and salinity), ozone and intense light.

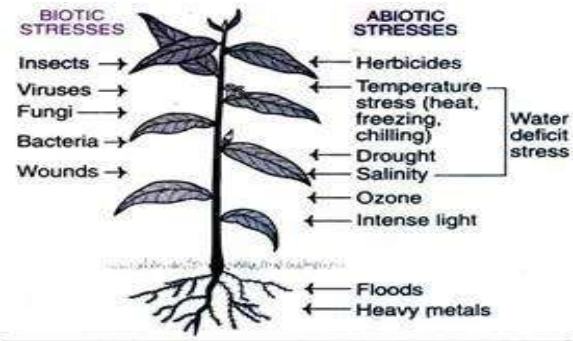


Fig. 50.1 : Biotic and abiotic stresses that affect plant growth, development and yield.

Almost all the stresses, either directly or indirectly, lead to the production of reactive oxygen species (ROS) that create oxidative stress to plants. This damages the cellular constituents of plants which is associated with a reduction in plant yield.

POSSIBLE QUESTIONS

8 MARKS

1. Write a note on applications of transgenic plants
2. Elaborate in detail on genetic engineering of plants.
3. Write a note on herbicide resistance plant production.
4. Discuss about biolistic methods in detail.
5. Explain in detail about the requirements of plant tissue culture and callus culture.
6. Elaborate in detail about plant transformation using genetic engineering.
7. Give a detailed account on media composition, nutrients and growth regulators for the plant tissue culture.
8. Explain the production of herbicide resistance plant and applications of transgenic plants.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
III-B.Sc., BIOCHEMISTRY
15BCU603A –CORE ELECTIVE II-INTRODUCTION TO BIOTECHNOLOGY
MULTIPLE CHOICE QUESTIONS

UNIT-V

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	A --------- measure oxygen consumption rates of low respiring and shear-sensitive cell suspensions	respiro plunger	respirometer	Plunger	None of the above	**respirometer**
2	Which of the following is the immortal cell line	Normal fibroblast	Non malignant epithelial cells	Haematopoietic cells	Malignant cells	**Malignant cells**
3	Animal tissues chopped up to ---------- pieces for explant preparation	1mm	0.1mm	0.01mm	$2mm^2$	**1mm**
4	Which of the following is not a serum constituent	Binding proteins	Cholesterol	Fe and Zn	Mg and Cu	**Mg and Cu**
5	Which of the following is the growth factor that is present in animal cell culture media	Interleukin I	Stem cell factor	Tumor necrosis factor	Scatter factor	**Scatter factor**
6	Scatter factor is called as --------	Transforming growth factor	Hepatocyte growth factor	Platelet derived growth factor	Nerve cell derived growth factor	**Hepatocyte growth factor**
7	Which of the following is the media additive?	Bovine pituitary extract	Endothelial cell growth supplement	Both a and b	MAB cells	**Both a and b**
8	The catalogue containing a complete list of 3,600 individual cell lines and hybridoma are called as	ATCC	CLSI	PTSP	All the above	**All the above**
9	Cells require attachment for growth is called	Anchorage dependent	Matrix dependent	Non adhesive dependent	Adhesive	**Anchorage dependent**
10	In animal cell culture subtrate surface should be treated by using ----------to improve cell attachment	Collagen	gelatin	poly d lysine	all the above	**all the above**
11	The animal cell culture vessels to be flushed out with ---------- Co2 gas prior to inoculation	5-10%	90-95%	95%	5-15%	**5-10%**
12	Calcium is reduced in suspension culture in order to	maximize cell aggregation	maximize cell attachment	maximize cell proliferation	Both a and b	**Both a and b**
13	Accumulation of lactic acid in animal tissue culture medium implies--------	improper citric acid cycle	improper glycolysis	both a and b	none of the above	**improper citric acid cycle**
14	Media with reduced serum concentration is called as	Holding media	Selective media	Complex media	Minimal media	**Holding media**
15	Vaccines and interferons are produced by -------- mode of tissue culture technique	Batch Culture	Suspension in bioreactors	Fiber reactor	Rotation cultures	**Suspension in bioreactors**
16	1979 who developed microcarriers of **reduced ion exchange capacity**	Levine et al	Gebb *et al*	van Wezel	Balin *et al*	**Levine et al**
17	Whole embryos commonly are used to establish ----------	Primary cell cultures	Established cultures	Organ cultures	Tissue cultures	**Primary cell cultures**
18	Protease is used to destroy the ---------- that normally interconnect cells	matrix in junctions	proteins in the junctions	carbohydrates in the junctions	All the above	**proteins in the junctions**
19	Highly differentiated cells are called ---------- because the plasma membrane is organized into at least two discrete regions	polarized	internal surfaces	external surfaces	nonpolarized	**polarized**
20	transport of nutrients from the cell to the blood and forms junctions with adjacent cells and the underlying extracellular matrix called	the basal lumen	the basal lamina	the basal mucoplasm	the basal epithelium	**the basal lamina**
21	Growth factors and mitogens factors omitted in ---------- media	Holding media	Serum free media	Complex media	Minimal media	**Serum free media**
22	---------- act as a major source of animal tissue contamination	CO2 incubator	Humidified incubator	O2 incubator	Aerated incubator	**Humidified incubator**
23	The term cell culture refers to	Cells taken from cell line	Cells taken from original tissue	Dispersed cells	Established cells	**Cells taken from original tissue**
24	Transformed cells derived from a single parental cell are called	Hybrid line	Cell line	Infinite cells	Finite cells	**Cell line**
25	Commercially available Microcarriers made by materials like	DEAE Sephadex	Cellulose	Glass and gelatin	All the above	**All the above**
26	Hollow fiber reactor consist of ---------- it helps "fibers", cells to grow	Semi-permeable membranes	Definite cut-off cells	Nutrients into the lumen	Metabolic by-products	**Semi-permeable membranes**
27	A multicellular organism that carries a specific genetic change in each cell because of an intervention at the fertilized egg stage is a	Transversion	Transition	Transgenic	Transforming	**Transgenic**
28	Genetic engineering manipulates gene products at the level of the	Protein	Amino acid	DNA	RAA	**DNA**

29	A knockout mouse is so named because	it contains aggression genes	it contains multiple copies of a human gene	it contains an activated human gene	none of the above	**it contains an activated human gene**
30	The first patent for a transgenic organism was awarded in 1988. Which organism was patented?	a yeast used in industrial processes	a bacterium able to metabolize components of crude oil	a mouse that manufactures human protein in its milk	life forms cannot be patented	**a mouse that manufactures human protein in its milk**
31	A cDNA version of a gene includes	codons for a mature mRNA	sequences corresponding to promoters	sequences corresponding to introns	both b and c	**codons for a mature mRNA**
32	Which of the following transgenic pharming products is incorrectly paired with the host organism that produces it?	hemoglobin – rabbit	human growth hormone-rat	lactoferrin – cow	alpha-1-antitrypsin – sheep	**hemoglobin – rabbit**
33	---------- Type of cultures should be kept in elevated O2 chamber	monolayer	keratinocyte	Embryonic cells	None of the options	**Embryonic**
34	Primary cells culture contains ---------- type of cells	Homogenous cells	Heterogeneous cells	Tumor cells	All the above	**Heterogeneous cell**s
35	---------- type of cells do not divide in invitro and can only used as primary culture	Macrophages	Neurons	Both a and b	Epidermal	**Both a and b**
36	The fusion of two cells that are genetically different yields a hybrid cell called a	Heterokaryon	Zygote	Gametes	Heterozygotes	**Heterokaryon**
37	The medium most often used to select hybrid cells is called	TMP medium	HAT medium	EPE medium	RPMI1640 medium	**HAT medium**
38	The genetic changes that allow these cells to grow indefinitely are collectively called	*oncogenic* transformation	*oncogenically transformed*	Simply *transformed*	All the above	***oncogenic* transformation**
39	The process of ____ involves the introduction of a gene into a cell where it exchanges places with its counterpart in the host cell	Transgenic technology	Gene targeting	Knockout technology	Recombinant DNA technology	**Gene targeting**
40	Which of the following gene-targeted mice have shown that a gene product once thought important is not vital to survival?	Neurofibromatosis	X collagen	SCID	sickle-cell	**X collagen**
41	The ----------grow human embryonic stem cells in the absence of mouse-derived "feeder" cells	nature research Institute	renke research Institute	Atl research Institute	WiCell research Institute	**WiCell research Institute**
42	In which stage of development does a zygote go through the structural and functional specialization of groups of cells?	growth	differentiation	morphogenesis	fertilization	**differentiation**
43	What is fertilization	The fusion of male and female gametes	The division of the zygote into a larger and larger number of smaller cells.	The continued division of cells that move inward to form three cellular layers	The development of pattern, shape, and f	**The fusion of male and female gametes**
44	The term for eggs that have only a small amount of yolk that are evenly distributed	holoblastic	telolecithal	isolecithal	morula	**isolecithal**
45	Which of the following are characteristics of reptile eggs	protective shells	large amount of yolk	intricate membrane system	all the above	**all the above**
46	Which structure in the bird egg functions to remove waste	allantois	allontois	chorion	all the above	**allantois**
47	Which structure in the bird egg corresponds to the 'egg white' used in cooking?	Albumin	shell	Yolk	Nucleous	**Albumin**
48	Gene targeting is done on a(n)	sperm cell	egg cell	fertilized ovum	early embryonic cell	**ear**l**y embryonic cell**
49	Human proteins can be produced in the milk or semen of farm animals. True or false?	TRUE	false, proteins cannot be produced in milk	false, proteins cannot be produced in semen	false, animals are not used for protein production	**TRUE**
50	____ consist of recombinant cells containing different fragments of a foreign genome	DNA probes	Homologous recombinants	Genomic libraries	Knockout organisms	**Genomic libraries**
51	____ are used to select genes of interest from a genomic library	Restriction enzymes	Cloning vectors	DNA probes	Gene targets	**DNA probes**
52	Hollow sphere presenting cell is called	morula	Blastula	Blastocoel	Blastomere	**Blastula**
53	Which of the following is a germ layer formed during gastrulation	ectoderm	endoderm	mesoderm	All the above	**All the above**
54	Because the fertilized egg of frogs has most of the yolk at the _____ pole, it is said to be -----	animal, telolecithal	animal, isolecithal	vegetal, telolecithal	vegetal, isolecithal	v**egetal, telolecithal**
55	Unlike the sea star gastrula, the frog does not invaginate, but produces a----------	blastopore	gray crescent	blastocoel	primitive streak	**blastopor**e
56	For the same gene, it is possible to patent	expressed sequence tags	cDNA	SNPs	All the above	**All the above**
57	Which gene transfer technique involves the use of a fatty bubble to carry a gene into a somatic cell	Electroporation	liposome transfer	microinjection	particle bombardment	**liposome transfer**
58	Naked DNA	is free of nucleic acids	is free of the cell	is free of protein	contains just the sugar-phosphate backbone	**is free of protein**

59	A fertilized egg undergoes cell division without further growth in the process called	gastrulation	cleavage	differentiation	morphogenesis	**cleavage**
60						
61	Which of these best represents development?	cells divide and get larger	cells become specialized in structure and function	Both a & b	Cells become dead	**Both a & b**

UNIT –V
SYLLABUS

Genetic engineering of animals – methodology-production of transgenic mice (with reference to insulin only). Knock out mice, Applications of Transgenic animals. Animal biotechnology-Artificial insemination and embryo transfer. *In vitro* fertilization (IVF). Animal cell culture-Facilities and culture media for animal cell culture. Primary cell culture techniques-cell separation and monolayer culture. Cell lines.Recombinant proteins from cell cultures: interferons, viral vaccines.Gene therapy.

Genetic engineering of animals – methodology

For practical reasons, i.e., their small size and low cost of housing in comparison to that for larger vertebrates, their short generation time, and their fairly well defined genetics, mice have become the main species used in the field of transgenics.

The three principal methods used for the creation of transgenic animals are DNA microinjection, embryonic stem cell-mediated gene transfer and retrovirus-mediated gene transfer.

a) DNA microinjection.

This method involves the direct microinjection of a chosen gene construct (a single gene or a combination of genes) from another member of the same species or from a different species, into the pronucleus of a fertilized ovum. It is one of the first methods that proved to be effective in mammals (Gordon and Ruddle, 1981). The introduced DNA may lead to the over- or under-expression of certain genes or to the expression of genes entirely new to the animal species. The insertion of DNA is, however, a random process, and there is a high probability that the introduced gene will not insert itself into a site on the host DNA that will permit its expression. The manipulated fertilized ovum is transferred into the oviduct of a recipient female, or foster mother that has been induced to act as a recipient by mating with a vasectomized male.

A major advantage of this method is its applicability to a wide variety of species.

b) Embryonic stem cell-mediated gene transfer.

This method involves prior insertion of the desired DNA sequence by homologous recombination into an in vitro culture of embryonic stem (ES) cells. Stem cells are undifferentiated cells that have the potential to differentiate into any type of cell (somatic and germ cells) and therefore to give rise to a complete organism. These cells are then incorporated into an embryo at the blastocyst stage of development. The result is a chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knock-out method.

This technique is of particular importance for the study of the genetic control of developmental processes. This technique works particularly well in mice. It has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination.

c) Retrovirus-mediated gene transfer.

To increase the probability of expression, gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

For any of these techniques the success rate in terms of live birth of animals containing the transgene is extremely low. Providing that the genetic manipulation does not lead to abortion, the result is a first generation (F1) of animals that need to be tested for the expression of the transgene. Depending on the technique used, the F1 generation may result in chimeras. When the transgene has integrated into the germ cells, the so-called germ line chimeras are then inbred for 10 to 20 generations until homozygous transgenic animals are obtained and the transgene is present in every cell. At this stage embryos carrying the transgene can be frozen and stored for subsequent implantation.

Production of transgenic mice

The Transgenic Mouse:

It is designed to support investigators doing biology of aging research by creating mice that have been genetically altered by either inserting a new gene or removing a normal gene.

This method has become one of the most exciting approaches of discovering the functions and interactions of genes in mammals. At the University Of Washington, Nathan Shock Center, this transgenic technology is used to develop new animal models for studying genetic mechanisms of the aging process (Fig. 18.4).

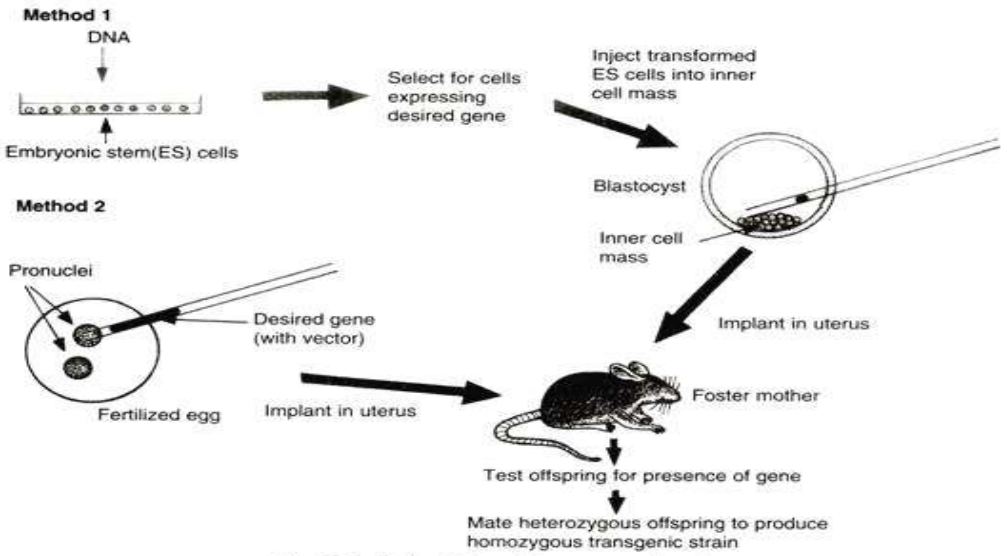


Fig. 18.4. Method of producing transgenic mice.

Method of producing transgenic mice

During the previous year, transgenic mouse production has focused on constructs with enhanced defense against free radical injury in aging (e.g., catalase, superoxide dismutase, glutathione S-transferase), Werner Syndrome, adult onset diabetes, Alzheimer's disease, thrombospondin, and rheumatoid arthritis in aging. Almost 4000 embryos, mainly of the C57BL/6 ingred strain, have been transferred, 498 pups analyzed and at least 40 contained the integrated construct.

The creation of "transgenic" animals that make a specified gene product presents a spectrum of opportunities for basic studies in molecular pathogenesis and pre-clinical investigations applicable to a wide variety of medical problems of aging. An additional gene transfer technology developed in the 1980's involved the use of stem cells from the early embryo, so-called embryonic stem (ES) cells. The capacity of ES cells to undergo differentiation makes them useful for investigating the effects of genetic modifications of either the gain of function or loss of function.

Recombinant insulin

Insulin, synthesized by the b-cells of the islets of Langerhans in the pancreas, controls the level of glucose in the blood. An insulin deficiency manifests itself as diabetes mellitus, a complex of symptoms which may lead to death if untreated. Fortunately, many forms of diabetes can be alleviated by a continuing program of insulin injections, thereby supplementing the limited amount of hormone synthesized by the patient's pancreas. The insulin used in this treatment was originally obtained from the pancreas of pigs and cows slaughtered for meat production. Although animal insulin is generally satisfactory, problems may arise in its use to treat human diabetes. One problem is that the slight differences between the animal and the human proteins can lead to side effects in some patients. Another is that the purification procedures are difficult, and potentially dangerous contaminants cannot always be completely removed. Insulin displays two features that facilitate its production by recombinant DNA techniques.

The first is that the human protein is not modified after translation by the addition of sugar molecules (p. 236): recombinant insulin synthesized by a bacterium should therefore be active. The second advantage concerns the size of the molecule. Insulin is a relatively small protein, comprising two polypeptides, one of 21 amino acids (the A
chain) and one of 30 amino acids (the B chain; Figure 14.1). In humans these chains are synthesized as a precursor called preproinsulin, which contains the A and B segments linked by a third chain (C) and preceded by a leader sequence. The leader sequence is removed after translation and the C chain excised, leaving the A and B polypeptides linked to each other by two disulphide bonds.

Several strategies have been used to obtain recombinant insulin. One of the first projects, involving synthesis of artificial genes for the A and B chains followed by production of fusion proteins in *E. coli*, illustrates a number of the general techniques used in recombinant protein production.

Synthesis and expression of artificial insulin genes

In the late 1970s, the idea of making an artificial gene was extremely innovative. Oligonucleotide synthesis was in its infancy at that time, and the available methods for making

artificial DNA molecules were much more cumbersome than the present-day automated techniques. Nevertheless, genes coding for the A and B chains of insulin were synthesized as early as 1978.

The procedure used was to synthesize trinucleotides representing all the possible codons and then join these together in the order dictated by the amino acid sequences of the A and B chains. The artificial genes would not necessarily have the same nucleotide sequences as the real gene segments coding for the A and B chains, but they would

still specify the correct polypeptides. Two recombinant plasmids were constructed, one carrying the artificial gene for the A chain, and one the gene for the B chain. In each case the artificial gene was ligated to a *lacZ′*reading frame present in a pBR322-type vector (Figure 14.2a). The insulin genes were therefore under the control

of the strong *lac* promoter (p. 231), and were expressed as fusion proteins, consisting of the first few amino acids of b-galactosidase followed by the A or B polypeptides (Figure 14.2b). Each gene was designed so that its b-galactosidase and insulin segments were separated by a methionine residue, so that the insulin polypeptides could be

cleaved from the b-galactosidase segments by treatment with cyanogen bromide (p. 233). The purified A and B chains were then attached to each other by disulphide bond formation in the test tube.

The final step, involving disulphide bond formation, is actually rather inefficient. A subsequent improvement was to synthesize not the individual A and B genes, but the entire proinsulin reading frame, specifying B chain–C chain–A chain (see Figure 14.1). Although this is a more daunting proposition in terms of DNA synthesis, the prohormone

has the big advantage of folding spontaneously into the correct disulphide-bonded structure. The C chain segment can then be excised relatively easily by proteolytic cleavage.

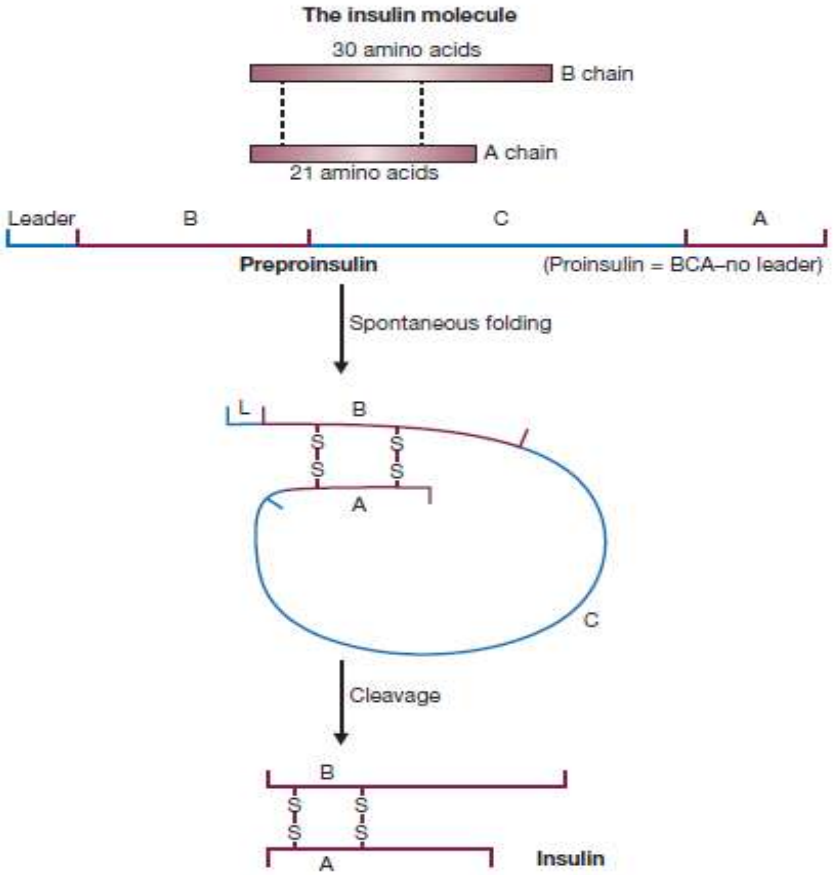


Fig: The structure of the insulin molecule and a summary of its synthesis by processing from preproinsulin

Knock out mice

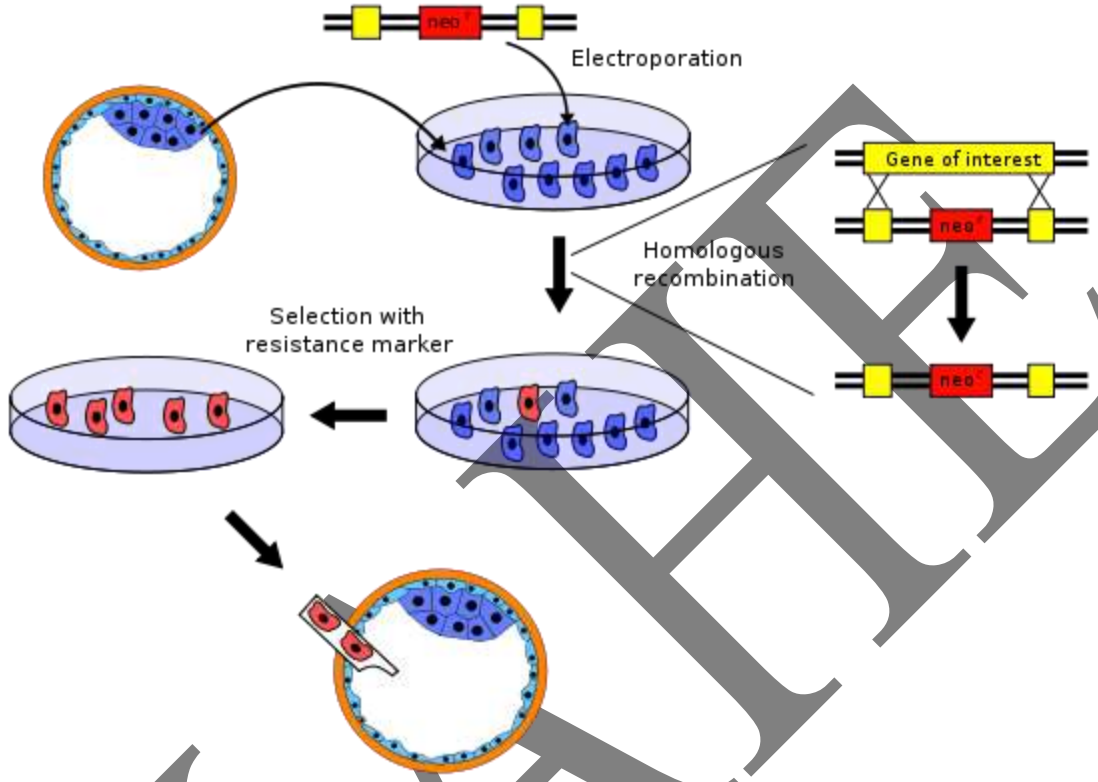
A knockout mouse or knock-out mouse is a genetically modified mouse (*Mus musculus*) in which researchers have inactivated, or "knocked out", an existing gene by replacing it or disrupting it with an artificial piece of DNA. They are important animal models for studying the role of genes which have been sequenced but whose functions have not been determined. By causing a specific gene to be inactive in the mouse, and observing any differences from normal behaviour or physiology, researchers can infer its probable function.

Use

Knocking out the activity of a gene provides information about what that gene normally does. Humans share many genes with mice. Consequently, observing the characteristics of knockout mice gives researchers information that can be used to better understand how a similar gene may cause or contribute to disease in humans. Examples of research in which knockout mice have been useful include studying and modeling different kinds of cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson's disease. Knockout

mice also offer a biological and scientific context in which drugs and other therapies can be developed and tested. Millions of knockout mice are used in experiments each year.

Procedure

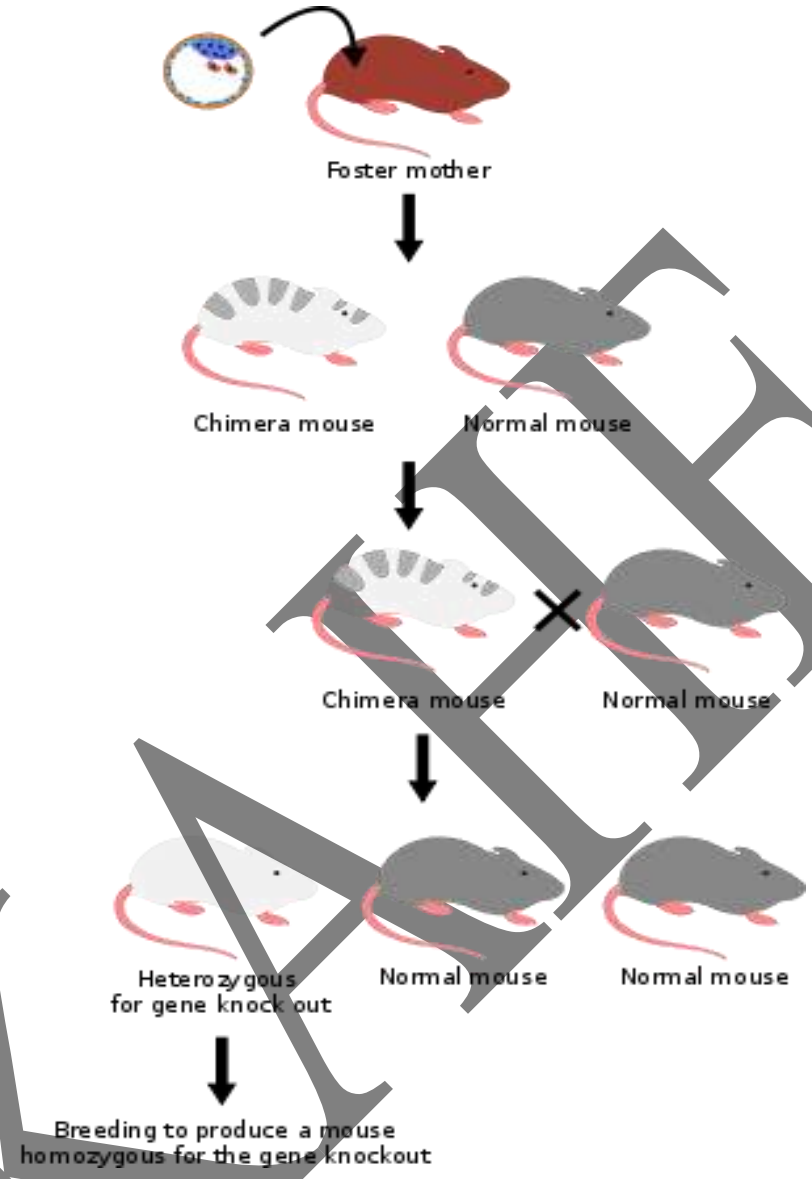


There are several variations to the procedure of producing knockout mice; the following is a typical example.

1. The gene to be knocked out is isolated from a mouse gene library. Then a new DNA sequence is engineered which is very similar to the original gene and its immediate neighbour sequence, except that it is changed sufficiently to make the gene inoperable. Usually, the new sequence is also given a marker gene, a gene that normal mice don't have and that confers resistance to a certain toxic agent (e.g., neomycin) or that produces an observable change (e.g. colour or fluorescence). In addition, a second gene, such as herpes tk+, is also included in the construct in order to accomplish a complete selection.
2. Embryonic stem cells are isolated from a mouse blastocyst (a very young embryo) and grown *in vitro*. For this example, we will take stem cells from a white mouse.
3. The new sequence from step 1 is introduced into the stem cells from step 2 by electroporation. By the natural process of homologous recombination some of the electroporated stem cells will incorporate the new sequence with the knocked-out gene into their chromosomes in place of the original gene. The chances of a successful

recombination event are relatively low, so the majority of altered cells will have the new sequence in only one of the two relevant chromosomes – they are said to be heterozygous. Cells that were transformed with a vector containing the neomycin resistance gene and the herpes tk+ gene are grown in a solution containing neomycin and Ganciclovir in order to select for the transformations that occurred via homologous recombination. Any insertion of DNA that occurred via random insertion will die because they test positive for both the neomycin resistance gene and the herpes tk+ gene, whose gene product reacts with Ganciclovir to produce a deadly toxin. Moreover, cells that do not integrate any of the genetic material test negative for both genes and therefore die as a result of poisoning with neomycin.

4. The embryonic stem cells that incorporated the knocked-out gene are isolated from the unaltered cells using the marker gene from step 1. For example, the unaltered cells can be killed using a toxic agent to which the altered cells are resistant.
5. The knocked-out embryonic stem cells from step 4 are inserted into a mouse blastocyst. For this example, we use blastocysts from a grey mouse. The blastocysts now contain two types of stem cells: the original ones (from the grey mouse), and the knocked-out cells (from the white mouse). These blastocysts are then implanted into the uterus of female mice, where they develop. The newborn mice will therefore be chimeras: some parts of their bodies result from the original stem cells, other parts from the knocked-out stem cells. Their fur will show patches of white and grey, with white patches derived from the knocked-out stem cells and grey patches from the recipient blastocyst.
6. Some of the newborn chimera mice will have gonads derived from knocked-out stem cells, and will therefore produce eggs or sperm containing the knocked-out gene. When these chimera mice are crossbred with others of the wild type, some of their offspring will have one copy of the knocked-out gene in all their cells. These mice will be entirely white and are not chimeras, however they are still heterozygous.
7. When these heterozygous offspring are interbred, some of their offspring will inherit the knocked-out gene from both parents; they carry no functional copy of the original unaltered gene (i.e. they are homozygous for that allele).



Applications of transgenic animals

The animal whose genetic material is taken from another organism is called as transgenic animal. The artificial genes are inserted in the germline cells of the organisms so they pass from one generation to the other. First transgenic animal was mouse which is the most important animal for doing experiments in the laboratory. But after that many other animals were also genetically modified through genetic engineering techniques for example rabbit, pig, sheep and other cattle. There are two reasons due to which the animals are produced through transgenic techniques:

1) There is one reason of producing transgenic animals that they can be beneficial economically. For example genetically modified cows give more milk then other cows and their milk contains human proteins which can be used to treat the disease of emphysema in humans.

2) Some transgenic animals are used as models for detecting, diagnosing and treating different diseases. For example most common laboratory animals are mice and rabbits. Scientists of Harvard produced a mouse carrying the genes which help in development of cancer. They named the mouse oncomouse or Harvard mouse.

Applications of Transgenic Animals in Medicine

We can say that medicine is one field which has took benefit of transgenic animals most. Every year people die of kidney failure, heart failure and due to other organs failure. But now scientists have succeeded in producing transgenic animals like pigs which carry the organs needed for humans. Pig is responsible for providing the organs to the humans and these organs can be inserted into the human body through transplantation. There are chances that pig protein might hinder organ transplantation but scientists are working on this issue and are thinking of replacing this protein with the human protein. United Kingdom is in the need of organs for its patients and according to the survey, almost 5000 organs are needed to be transplanted in patients with different diseases.

Transgenic animals have already helped in developing certain growth hormones. For this purpose, special genes are inserted in the milk of the cow, which increase the production of the milk. Similarly various genetic and hereditary diseases like cystic fibrosis and phenylketonuria can be treated by manufacturing milk which is developed with transgenesis.

Human gene therapy has provided opportunities for medicine that in a certain genetic or acquired disease, damaged genes can be replaced with the healthy genes and their function is secured.

Agriculture

Agriculture is another field which has taken advantage of transgenic animals. In livestock, when transgenic animals are bred with the normal animals, the generation produced contains healthy organs, meat and milk. Transgenic techniques have made quick production of animals while traditional methods of animals breeding are time taking. Quality of the animals also increases and they are the source of providing good quality food to humans in the form of milk and meat.

Industry

Two scientists of Canada succeeded in inserting spider genes in the goats who gave milk. The goats produced silk with the milk in large amounts. It was a good source of economy for the industrialists. Polymer strands of the silk were extracted from the milk and light threads were made. This thread could be used in the making uniforms of soldiers and also the tennis rockets.

Animal biotechnology is a branch of biotechnology in which molecular biology techniques are used to genetically engineer (i.e. modify the genome of) animals in order to improve their suitability for pharmaceutical, agricultural or industrial applications.

Animal biotechnology

Artificial insemination

A male animal produces millions of sperms daily. Theoretically, it can inseminate females regularly and produce several offsprings. This excess capacity of male has been utilized through developing new technologies for artificial insemination which can be said as the first animal biotechnology.

The most effective factor that has increased the productivity of cattle is the artificial insemination. However, the breeder must replace the nature through artificial insemination if he ensures about the ovulation of female in herd together at a time. In contrast, if a breeder awaits for female to ovulate and then inseminate separately, the importance and economic significance of artificial insemination get reduced. Therefore, through artificial insemination technology increased number of females can be inseminated by a male.

Semen and its storage

In addition methods have been developed to produce semen from male by ejaculation. Semen ejaculate is collected and diluted (extended). Sperm motility and their number per milliliter are examined under the microscope. About 0.2 ml bull semen contains about 10 million motile sperms. The diluted sperms may be used fresh within a few days or cryopreserved at -196°C by using liquid nitrogen (for detailed description see Cryobiology). The cryopreserved semen can be stored for a long time and easily transported across the states or countries. Thus, cryopreserved semen of a single male is capable of inseminating thousands of females of a country or other countries. For example, one ejaculate semen of a bull is sufficient to inseminate about 500 cows.

Ovulation control

In many animals it is difficult to find out oestrous (sexual heat) in animals because it persists only for a few hours and occurs mostly at night. After ovulation (which is indicated by oestrous) females are inseminated. But in a herd it would be economical, easy and simplified management if females are inseminated at a times. However, it is possible only when all the female ovulate at a time; in practice it is not possible to get synchronization of oestrous. Moreover, it is possible to bring about ovulation in about 80 per cent of females by using hormones, for example progesteron and/or prostaglandin. These hormones regulate ovulation cycle of female and result in total synchrony of oestrous.

Sperm sexing

Sperms are produced in the testes of males and ova in female's ovaries. Sperms and ova contain half of chromosomes as compared to somatic cells. An ovum possesses autosomes and one X chromosome. Similarly, a sperm contains autosomes and one Y chromosome. In animals sex is determined genetically i.e. by sex chromosomes. X chromosome determines femaleness and Y maleness. All the ova contain X chromosome, whereas a sperm consists of either X or Y chromosomes. One sperm ejaculate contains half X and half Y chromosomes.

Embryo transfer

In 1890, the first case of developing pregnancy in rabbits through embryo transfer is known in literature. During 1930s the same method was used in goat and sheep. In catties cases of embryo transfer were reported after 1950 (BIOTOL series, 1992). Embryo transfer method cannot be used widely because of its high cost, technical difficulties and limited supply of embryo from superovulated donors. The embryo develops in foster mother (recipient) which simply acts as incubator and does not make any genetic contribution to the offspring.

Secondly, ruminant female carries one pregnancy at a time as only one egg is produced and fertilized with male's sperm. Thus, there is a chance for increasing the number of egg production at a time and transfer of fertilized eggs i.e. embryo into uterus of less important foster mothers other than original female in farm animal.

Multiple ovulation (Superovulation)

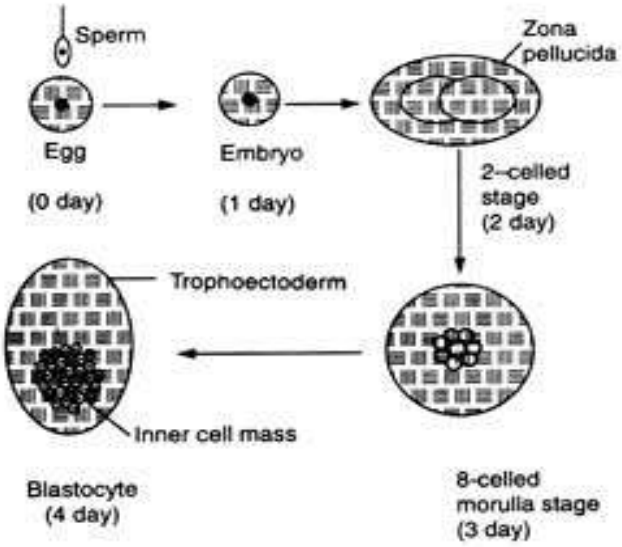
The reproductive cycle of ruminant female is such that the ovarian follicle of a non-pregnant female matures and releases single egg at a time. The time of ovulation differs in different animals, for example 21 days in cows and horse, 16 days in sheep and goats, etc. Normally ovulation occurs as a result of circulation of gonadotropic hormone. But by increasing the concentration of hormone the number of egg production gets increased. In well managed domestic catties 8-10 eggs are superovulated; the number may go to 60. However, this depends on health, nutrition, breed of animals and environment in which they live.

Price (1991) has reviewed the current practice of multiple ovulation and superovulation. This technique has spread from cattle and sheep to goats, horses and deers. He has emphasized that (i) general selection for increased litter size is useful only in sheep, (ii) the gonadotropic hormone induces superovulation in goats, sheeps and cattle but the response varies so much, and (iii) immunization against ovarian steroid hormones can increase litter size in sheep. Therefore, different molecular forms of follicle stimulation hormones (FSH) should be characterized in the farm animals. Misra et al. (1990) have reported multiple ovulation and embryo transfer in Indian buffalo (Bubalus bubalis).

After injecting the gonadotrophin the females are induced for superovulation. During follicular phase (second phase of oestrous cycle) about 20 ovarian follicles are induced. Eventually these grow and filled with fluid. The space of follicle which is filled with fluid is called antrum, and such follicles as antral follicles. In normal course, only one follicle develops and releases one egg after maturation. However, before ovulation the follicles laying against surface of ovary looks large sized (8 mm in sheep and pigs, 15 mm in cattle). Therefore, immature oocytes from follicles of donor females are recovered surgically by using laparoscope.

The females to be superovulated are frequently injected with prostaglandin F2a (PGF2a) so that synchronized oestrous could develop in them. After 10 days of oestrous they are injected with hormone FSH up to 4 days followed by PGF2a treatment so that oestrous may be maintained.

The FSH treatment induces superovulation. The females are artificially inseminated. The eggs are fertilized. After fertilization embryos undergo developmental stages.



Multiple Ovulation with Embryo Transfer (MOET)

After 6-8 days of fertilization (for sheep and goat) the embryos are recovered. During this stage embryos have come to morula or blastula atage and remain in female's oviduct. In cattle the embryos are recovered without surgery by inserting a catheter into oviduct. A saline solution is drained in oviduct and embryos flushed out through catheter into a storage bottle. The oviduct of sheep is small, therefore, it is exposed surgically and embryos are recovered by syringe adopting the same method for cattle.

In storage bottle embryos settle down and solution decanted. Embryos with small volume of saline are poured into a Petri dish. Then they are examined under microscope. The identified embryos are transferred into synchronized recipient females (|as artificial insemination), when they are in 6-8 days of cycle of embryo development, stored and transported or manipulated as desired. In general, one superovulated female results into 5-6 progenies. In cattle 50-60 per cent of pregnancy can be achieved from embryo transfer method (Ebert, 1989).

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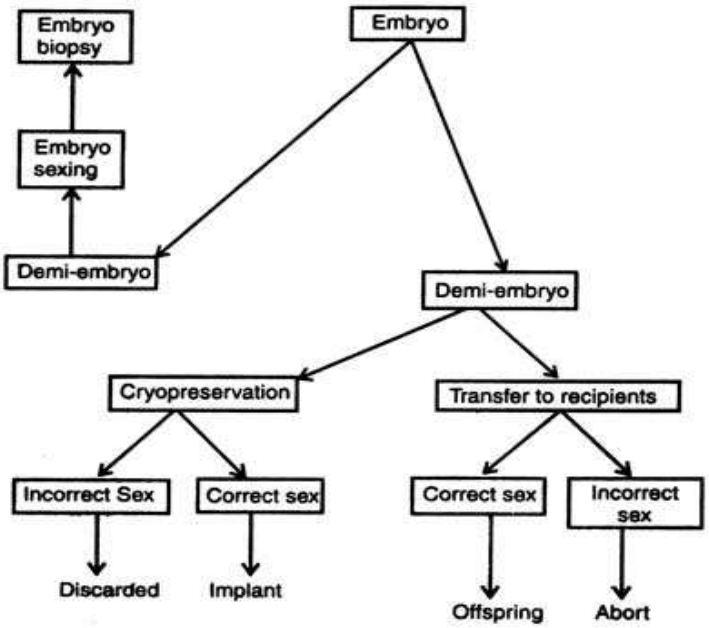
Embryo splitting (Demi-embryo)

Embryo of blastocyst stage is differentiated into two regions, trophoectoderm and inner cell mass (ICM).

Trophoectoderm is a single layer of trophoblast cells lining the inner side of zone surrounding the blastoceal (a fluid filled cavity). Zona becomes the foetal membrane bf placenta. The ICM is the mass of cells which develops into foetus.

The embryos of blastocyst stage can be split into equal two halves and transferred to females to produce identical twins. Thus, embryo splitting technology has increased the rate of pregnancy. To carry out embryo splitting, the blastocyst stage embryos are transferred for a few minutes into a cell culture medium consisting of hypertonic sucrose and bovine serum albumin. The medium of high osmotic strength enters into cell membrane of the embryo (zona pellucida) and cells contract due to exosmosis. The bovine albumin serum attaches to zona pellucida and provides negative charge to the embryo. Then it is transferred into a plastic Petri dish containing standard cell culture medium. The outer membrane of embryo is negatively charged and Petri dish positively. Embryo sticks to the surface of Petri dish due to development of electrostatic interaction of two charges. The Petri dish is kept on stage of an inverted microscope. A micromanipulator equipped with fine surgical blade is used to cut the embryo roughly into two halves with minimal damage to the cells. The hemispherical mass of half embryo, demi-embryo or semi-embryo reforms spheres.



However, it is not necessary for successful implantation of demi-embryo that it should be enclosed in a zona pellucida. The demi-embryos are transferred into oviduct of synchronized recipients as described for normal embryo transfer. At this stage it is very necessary to know the situation for successful embryo implantation that the wall of uterus of synchronized female recipient is waiting for embryo, and embryo is prepared to send chemical signals from the trophoblast (Ebert, 1989).

To increase the rate of pregnancy, the embryo can be cut into equal three or four pieces, and transplanted in synchronized females. But too small trophoblast will not induce the uterus for pregnancy. So far it is unknown how cells of ICM are required for successful pregnancy, but it is clear that increasing in embryo splitting will have less probability of pregnancy.

Embryo biopsy (removal of small number of cells for genetic analysis) should be combined with splitting so that the twins which will be produced should be identical and of known genotype. Biopsy is very necessary in breeding so that sex and genetic diseases could be detected. In case the embryo has any genetic disease, it can be prevented from implantation in recipient females.

Embryo sexing

Before the implantation of embryo its sex should be detected from the biopsy sample. The principle for sexing is very common. The presence of Y chromosome makes the offsprings male and that of X makes female. The second method is the use of polymerase chain reaction (PCR) machine in sex detection. PCR amplifies DNA sequence of Y chromosome and reaction products can be seen directly. Handy side et al. (1989) isolated single blastomere from early embryo from a womb, amplified DNA sequences of Y chromosome and carried out embryo sexing before implantation into uterus. It is true that the PCR was first commercially implemented for embryo sexing of livestock.

***In vitro* fertilization (IVF)**

The term in vitro means in glass or in artificial conditions, and IVF refers to the fact that fertilization of egg by sperm had occurred not in uterus but out side the uterus at artificially maintained optimum condition. In recent years the IVF technology has revolutionized the field of animal biotechnology because of production of more and more animals as compared to animal production through normal course. For example, an animal produces about 4-5 offsprings in her life through normal reproduction, whereas through IVF technology the same can produce 50-80 offsprings in her life. Therefore, the IVF technology holds a great promise because a large number of animals may be produced and gene pool of animal population can also be improved. In India M.L. Madan, an animal embryo-biotechnologist at National Dairy Research Institute, Karnal (Haryana) has got success in producing more calves in cows.

The IVF technology is very useful. It involves the procedure : (i) taking out the eggs from ovaries of female donor, (ii) in vitro maturation of egg cultures kept in an incubator, (iii) fertilization of the eggs in test tubes by semen obtained from superior male, and (iv) implantation of seven days old embryos in reproductive tract of other recipient female which acts as foster

mother or surrogate mothers. These are used only to serve as animal incubator and to deliver offsprings after normal gestation period. The surrogate mothers do not contribute any thing in terms of genetic make up since the same comes from the egg of donor mother and semen from artificial insemination.

***In vitro* Maturation (IVM) of oocytes**

The immature oocytes are incubated in vitro so that they can be mature. However, immature oocytes should be taken out from follicles because they cannot mature in it but degenerate. Therefore, full potential of superovulation and all the oocytes can be utilized by IVF technology. Moreover, metabolic and hormonal requirement for oocytes during IVM should be found out so that the present rate of maturation (20%) could be improved. In majority of cases ovarian follicles never reach maturity and degenerate due to unknown causes. Possibly there may be genetic defects associated with them.

Culture of *in vitro* fertilized embryos

IVF ofeggs is carried out in small droplets (microdroplets) of culture medium. Each microdroplet comprises of about 10 oocytes. The medium should be supplemented with penicillamine, hypotaurin, and epinephrine because they facilitate penetration of sperms into oocytes. Moreover, one dose of sperm is given that consists of about one million sperms per ml of medium. Thus, IVF embryo must be maintained at in vitro conditions for a few days so that it may develop into blastocyte. It takes about seven days for sheep and goats and eight days for cattle. There are many laboratories where about 60 per cent IVF embryos of catties are cultured to blastocyte stage.

The term delivery from cultured embryo is very low due to occurrence of high loss during first two months of pregnancy. This may be due to abortion of foetuses arising from the presence of genetic defects. It should be noted that before birth about 80 per cent genes play a key role in differentiation and development of foetuses. The oocytes which are forced to mature in vitro occasionally bears some defects. Some times environmental mutagenesis occurs in eggs, sperms or embryos. Artificial culture media should be improved as oxygen may have toxic effect. Therefore, gas atmosphere should be carefully controlled (Read and Smith, 1996).

Animal cell culture

Facilities and culture media for animal cell culture

Requirements for animal cell and tissue culture are the same as described for plant cell, tissue and organ culture (*In vitro* Culture Techniques: The Biotechnological Principles). Desirable requirements are (i) air conditioning of a room, (ii) hot room with temperature recorder, (iii) microscope room for carrying out microscopic work where different types of microscopes should be installed, (iv) dark room, (v) service room, (vi) sterilization room for sterilization of glassware and culture media, and (vii) preparation room for media preparation, etc. In addition the storage areas should be such where following should be kept properly : (i) liquids-ambient (4-20°C), (ii)

glassware-shelving, (iii) plastics-shelving, (iv) small items-drawers, (v) specialized equipments-cupboard, slow turnover, (vi) chemicals-sidled containers.

Substrates for Cell Growth

There are many types of vertebrate cells that require support for their growth in vitro otherwise they will not grow properly. Such cells are called anchorage-dependent cells. Therefore, a large number of substrates which may be adhesive (e.g. plastic, glass, palladium, metallic surfaces, etc.) or non-adhesive (e.g. agar, agarose, etc) types may be used as discussed below:

Plastic as a substrate. Disposable plastics are cheaper substrate as they are commonly made up of polystyrene. After use they should be thrown at proper place. Before use they are treated with gamma radiation or electric arc simply to develop charges on the surface of substrate. After cell growth its rate of proliferation should be measured. In addition, the other plastic materials used as substrate are teflon or polytetrafluoroethylene (PTFE), thermamox (TPX), polyvinylchloride (PVC), polycarbonate, etc. It should be noted that monolayer of cell must be grown. Moreover, plastic beads of polystyrene, sephadex and polyacrylamide are also available for cell growth in suspension culture.

Glass as a substrate. Glass is an important substrate used in laboratory in several forms such as test tubes, slides, coverslips, pipettes, flasks, rods, bottles, Petti dishes, several apparatus, etc. These are sterilized by using chemicals, radiations, dry beat (in oven) and moist heat (in autoclave).

Palladium as a substrate. For the first time palladium deposited on agarose was used as a substrate for growth of fibroblast and glia.

Culture Media

Culture of animal cells and tissue is rather more difficult than that of microorganisms and plants because the later synthesize certain chemical constituents from inorganic substances. However, the culture media provide the optimum growth factors (e.g. pH, osmotic pressure, etc) and chemical constituents (unlike microbes). There are two types of media used for culture of animal cell and tissue, the natural media and the synthesized media.

Natural media

Natural media are the natural sources of nutrient sufficient for growth and proliferation of animal cells and tissue. These are of three types : (i) coagulans or plasma clots (it is used since long time but now available in market in the form of liquid plasma kept in silicon ampoules or lyophilized plasma. Plasma may also be prepared in laboratory taking out blood from male fowl and adding heparin to prevent blood coagulation), (ii) biological fluid (it is obtained in the form of serum from human adult blood, placental, cord blood, horse blood, calf blood or in the form of biological fluids such as coconut water, amniotic fluid, pleural fluid, insect haemolymph serum, culture filtrate, aqueous humour (from eyes), etc. The most commonly used fluids are human placental, cord serum and foetal calf serum. Before use its toxicity should be checked, and (iii) tissue extract (extract from some tissues such as embryo, liver, spleen, leukocytes, tumour, bone

marrow, etc. are also used for culture of animal cells, where embryo extract is of most common use. Tissue extract should be used before a week or stored at 27°C.

Synthetic media

Synthetic media are prepared artificially by adding several nutrients (orgtmie and inorganic), vitamins, salts, O2 and CO2 gas phases, serum proteins, carbohydrates, cofactors, etc. However, different types of synthetic media may be prepared for a variety of cells and tissues to be cultured. It can be prepared for different functions. Basically, synthetic media are of two types, serum-containing media (i.e. the media containing serum) and serum-free media (i.e. media devoid of serum). Example of some of the media are: minimal essential medium (MEM) (Eagle, 1955), 199 (Morgan et al. 1950), CMRL 1066 (Parker et al, 1957), RPMI 1640 (Moore et al, 1967) and F12 (Ham, 1965). Some of these media are given in Table below.

Table: Chemical composition of different media (with serum) used for animal cell and tissue culture (quantities are in mg/l).

Chemical constituents	*Eagle's MEM*	*Dulbecco's modification*	*Ham's F12*
Amino Acids			
L-asparagine	126	84	211
L-cystine	24	48	-
L-glutamine	292	584	146
Glycine	-	30	7.5
L-histidine HC1.H_2O	42	42	21
L-isoleucine	52	42	21
L-leucine	52	105	13.1
L-lysine.HCl	73.1	146	36.5
L-methionine	15	30	4.48
L-phenylalanine	33	66	4.96
L-proline	-	-	34.5
L-serine	-	42	10.5
L-threonine	48	95	11.9
L-tryptophan	10	16	2.04
L-tyrosine	36	72	5.4
L-valine	47	94	11.7
Vitamins			
Biotin	-	-	0.0073
D-Ca-pantothenate	1	4	0.48
Choline chloride	1	4	14
Folic acid	1	4	1.3
Inositol	2	7.2	18

Nicotinamide	1	4	0.04
Pyridoxal.HCl	1	4	0.062
Riboflavin	0.1	0.4	0.038
Thiamine.HCl	-	-	1.36
Vitamin B12	-	-	1.36
Pyridoxin.HCl	-	-	0.062
Inorganic Salts			
$CaCl_2$ (anhydrous)	200	200	-
$CaCl_2.2H_2O$	-	-	44
$Fe(NO_3)_3.9H_2O$	-	0.1	-
KC1	400	400	221
$MgCl_2.6H_20$	-	-	122
$MgSO_4.7H_2O$	200	200	-
NaCl	6800	6400	7599
$NaHCO_3$	2200	3700	1176
$Na_2H_2PO_4.H_2O$	140	125	-
$Na_2HPO_4.7H_2O$	-	-	268
$CuSO_4.5H_2O$	-	-	0.00249
$FeSO_4.7H_2O$	-	-	0.834
$ZnSO_4.7H_2O$	-	-	0.863
Other Chemicals			
D-glucose	1000	4500	1802
Lipoic acid	-	-	0.21
Phenol red	10	15	12
Sodium pyruvate	-	110	110
Hypoxanthine	-	-	4.1
Linoleic acid	-	0.084	-
Putrescine.2HCl	-	-	0.161
Thymidine	-	-	0.73
CO_2 (gas phase)	5%	10%	5%

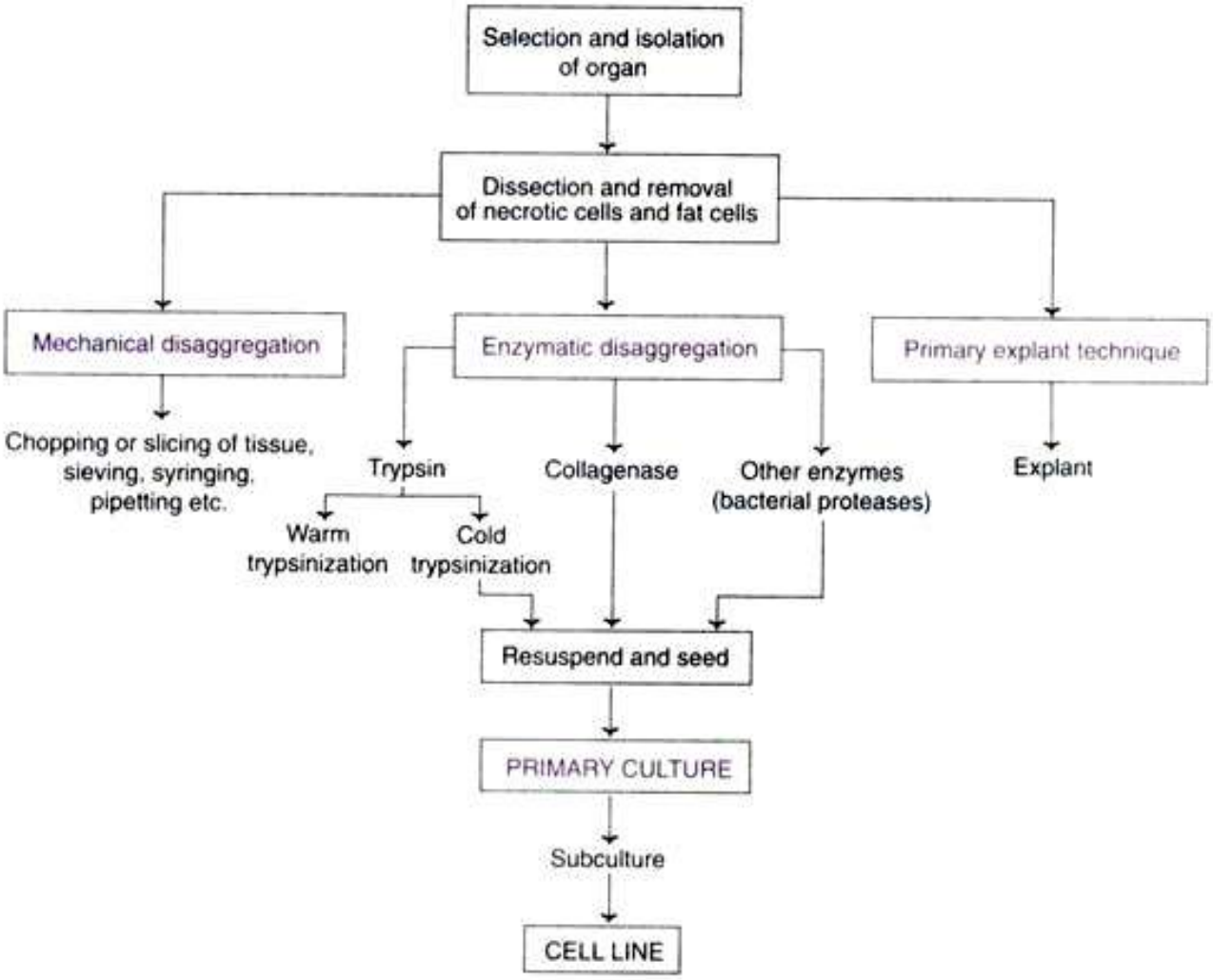
Primary Culture Techniques

Among the various techniques devised for the primary culture of isolated tissues, three techniques are most commonly used:

1. Mechanical disaggregation.

2. Enzymatic disaggregation.
3. Primary explant technique.

An outline of these techniques is depicted in Fig., and the procedures are briefly described:



1. Mechanical Disaggregation:

For the disaggregation of soft tissues (e.g. spleen, brain, embryonic liver, soft tumors), mechanical technique is usually employed. This technique basically involves careful chopping or slicing of tissue into pieces and collection of spill out cells.

The cells can be collected by two ways:

i. Pressing the tissue pieces through a series of sieves with a gradual reduction in the mesh size.

ii. Forcing the tissue fragments through a syringe and needle.

Although mechanical disaggregation involves the risk of cell damage, the procedure is less expensive, quick and simple. This technique is particularly useful when the availability of the tissue is in plenty, and the efficiency of the yield is not very crucial. It must however, be noted

that the viability of cells obtained from mechanical techniques is much lower than the enzymatic technique.

2. Enzymatic Disaggregation:

Enzymatic disaggregation is mostly used when high recovery of cells is required from a tissue. Disaggregation of embryonic tissues is more efficient with higher yield of cells by use of enzymes. This is due to the presence of less fibrous connective tissue and extracellular matrix. Enzymatic disaggregation can be carried out by using trypsin, collagenase or some other enzymes.

Disaggregation by trypsin:

The term trypsinization is commonly used for disaggregation of tissues by the enzyme, trypsin. Many workers prefer to use crude trypsin rather than pure trypsin for the following reasons:

i. The crude trypsin is more effective due to the presence of other proteases

ii. Cells can tolerate crude trypsin better.

iii. The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin. There are two techniques of trypsinization-warm trypsinization and cold trypsinization.

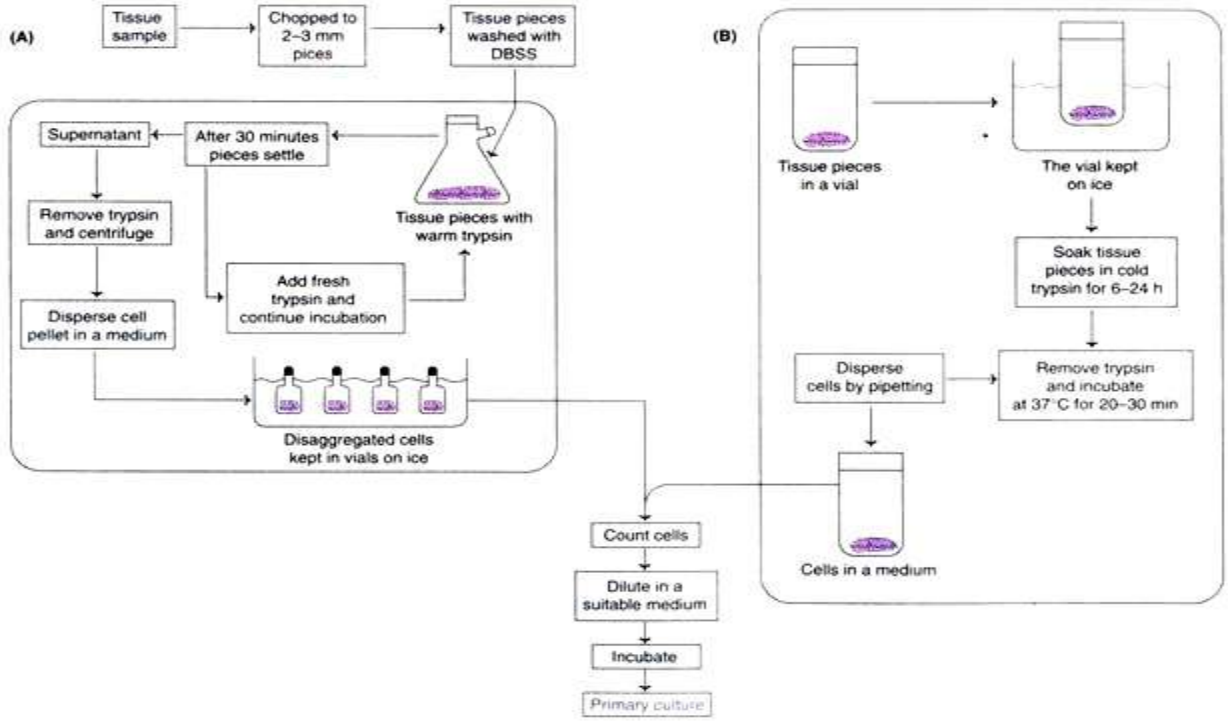


Fig. 36.2 : Preparation of primary culture by trypsin disaggregation (A) Warm trypsinization (B) Cold trypsinization (DBSS–Dissection basal salt solution).

Warm trypsinization (Fig. 36.2A):

This method is widely used for disaggregation of cells. The chopped tissue is washed with dissection basal salt solution (DBSS), and then transferred to a flask containing warm trypsin (37° C). The contents are stirred, and at an interval of every thirty minutes, the supernatant containing the dissociated cells can be collected. After removal of trypsin, the cells are dispersed in a suitable medium and preserved (by keeping the vial on ice).

The process of addition of fresh trypsin (to the tissue pieces), incubation and collection of dissociated cells (at 30 minutes intervals) is carried out for about 4 hours. The disaggregated cells are pooled, counted, appropriately diluted and then incubated.

Cold trypsinization (Fig. 36.2B):

This technique is more appropriately referred to as trypsinization with cold pre-exposure. The risk of damage to the cells by prolonged exposure to trypsin at 37°C (in warm trypsinization) can be minimized in this technique.

After chopping and washing, the tissue pieces are kept in a vial (on ice) and soaked with cold trypsin for about 6-24 hours. The trypsin is removed and discarded. However, the tissue pieces contain residual trypsin. These tissue pieces in a medium are incubated at 37°C for 20-30 minutes. The cells get dispersed by repeated pi-pettings. The dissociated cells can be counted, appropriately diluted and then used.

The cold trypsinization method usually results in a higher yield of viable cells with an improved survival of cells after 24 hours of incubation. This method does not involve stirring or centrifugation, and can be conveniently adopted in a laboratory. The major limitation of cold trypsinization is that it is not suitable for disaggregation of cells from large quantities of tissues.

Limitations of trypsin disaggregation:

Disaggregation by trypsin may damage some cells (e.g. epithelial cells) or it may be almost ineffective for certain tissues (e.g. fibrous connective tissue). Hence other enzymes are also in use for dissociation of cells.

Disaggregation by collagenase:

Collagen is the most abundant structural protein in higher animals. It is mainly present in the extracellular matrix of connective tissue and muscle. The enzyme collagenase (usually a crude one contaminated with non-specific proteases) can be effectively used for the disaggregation of several tissues (normal or malignant) that may be sensitive to trypsin.

Highly purified grades of collagenase have been tried, but they are less effective when compared to crude collagenase. The important stages in collagenase disaggregation, depicted in Fig. 36.3, are briefly described hereunder.

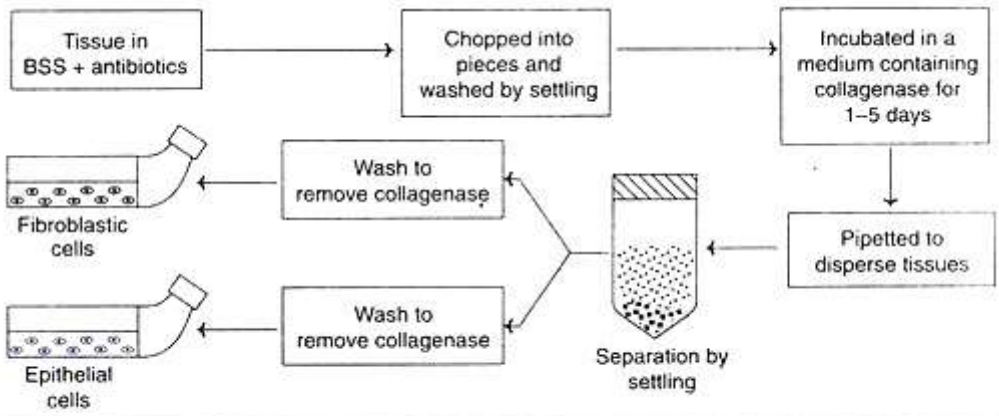


Fig. 36.3 : Important stages in collagenase disaggregation of tissue for primary culture (BSS–Basal salt solution).

The desired tissue suspended in basal salt solution, containing antibiotics is chopped into pieces. These pieces are washed by settling, and then suspended in a complete medium containing collagenase. After incubating for 1-5 days, the tissue pieces are dispersed by pipetting. The clusters of cells are separated by settling. The epithelial cells and fibroblastic cells can be separated.

Collagenase disaggregation has been successfully used for human brain, lung and several other epithelial tissues, besides various human tumors, and other animal tissues. Addition of another enzyme hyaluronidase (acts on carbohydrate residues on cell surfaces) promotes disaggregation.

Collagenase in combination with hyaluronidase is found to be very effective for dissociating rat or rabbit liver. This can be done by per-fusing the whole organ in situ. Some workers use collagenase in conjunction with trypsin, a formulation developed in chick serum, for disaggregation of certain tissues.

Use of other enzymes in disaggregation:

Trypsin and collagenase are the most widely used enzymes for disaggregation. Certain bacterial proteases (e.g. pronase, dispase) have been used with limited success. Besides hyaluronidase, neuraminidase is also used in conjunction with collagenase for effective degradation of cell surface carbohydrates.

3. Primary Explant Technique:

The primary explant technique was, in fact the original method, developed by Harrison in 1907. This technique has undergone several modifications, and is still in use. The simplified procedure adopted for primary explant culture is depicted in Fig. 36.4, and briefly described below.

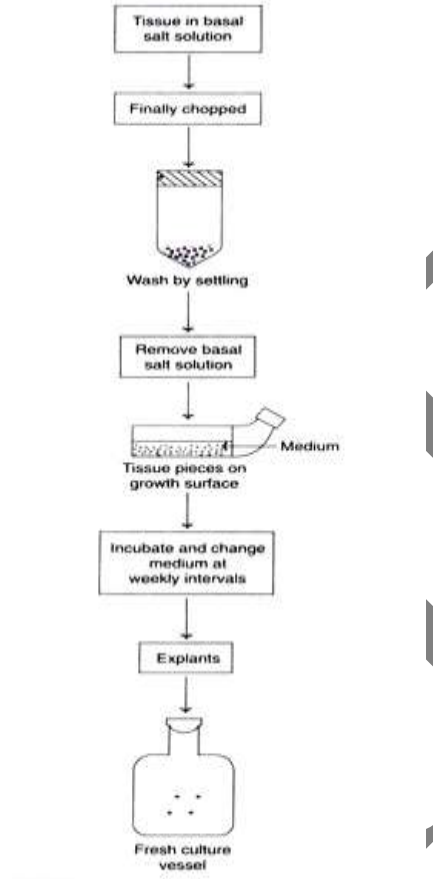


Fig. 36.4 : Primary explant technique for primary culture.

The tissue in basal salt solution is finely chopped, and washed by settlings. The basal salt solution is then removed. The tissue pieces are spread evenly over the growth surface. After addition of appropriate medium, incubation is carried out for 3-5 days. Then the medium is changed at weekly intervals until a substantial outgrowth of cells is observed. Now, the explants are removed and transferred to a fresh culture vessel.

The primary explant technique is particularly useful for disaggregation of small quantities of tissues (e.g. skin biopsies). The other two techniques mechanical or enzymatic disaggregation however, are not suitable for small amounts of tissues, as there is a risk of losing the cells.

The limitation of explant technique is the poor adhesiveness of certain tissues to the growth surface, and the selection of cells in the outgrowth. It is however, observed that the primary explant technique can be used for a majority of embryonic cells e.g. fibroblasts, myoblasts, epithelial cells, glial cells.

Separation of Viable and Non-Viable Cells:

It is a common practice to remove the nonviable cells while the primary culture is prepared from the disaggregated cells. This is usually done when the first change of the medium is carried out.

The very few left over non-viable cells get diluted and gradually disappear as the proliferation of viable cells commences.

Sometimes, the non-viable cells from the primary cultures may be removed by centrifugation. The cells are mixed with ficoll and sodium metrizoate, and centrifuged. The dead cells form a pellet at the bottom of the tube.

Medical Ethics and Safety Measures in Culture Techniques:

Since the culture techniques involve the use of animal or human tissues, it is absolutely necessary to follow several safety measures and medical ethics. In fact, in some countries there are established legislation/norms for selection and use of tissues in cultures. For example, in United Kingdom, Animal Experiments (Scientific Procedures) Act of 1986 is followed.

The handling of human tissues poses several problems that are not usually encountered with animal tissues. While dealing with fetal materials and human biopsies, the consent of the patient and/his or her relatives, besides the consent of local ethical committee is required. Further, taking any tissue (even in minute quantities) from human donors requires the full consent of the donor in a prescribed format.

The following issues need to be fully considered while dealing with human tissues:

1. The consent of the patient and/or relatives for using tissues for research purposes.
2. Ownership of the cell lines developed and their derivatives.
3. Consent for genetic modification of the cell lines.
6. Patent rights for any commercial use of cell lines.

In the general practice of culture techniques using human tissues, the donor and/or relatives are asked to sign a disclaimer statement (in a prescribed pro-forma) before the tissue is taken. By this approach, the legal complications are minimized.

Safety measures:

Handling of human tissues is associated with a heavy risk of exposure for various infections. Therefore, it is absolutely necessary that the human materials are handled in a biohazard cabinet. The tissues should be screened for various infections such as hepatitis, tuberculosis, HIV, before their use. Further, the media and apparatus, after their use must be autoclaved or disinfected, so that the spread of infections is drastically reduced.

COMMON CELL LINES

Human cell lines

DU145 (prostate cancer)
H295R (adrenocortical cancer)
HeLa (cervical cancer)
KBM-7 (chronic myelogenous leukemia)
LNCaP (prostate cancer)
MCF-7 (breast cancer)
MDA-MB-468 (breast cancer)

PC3 (prostate cancer)
SaOS-2 (bone cancer)
SH-SY5Y (neuroblastoma, cloned from a myeloma)
T47D (breast cancer)
THP-1 (acute myeloid leukemia)
U87 (glioblastoma)
National Cancer Institute's 60 cancer cell line panel (NCI60)

Primate cell lines

Vero (African green monkey Chlorocebus kidney epithelial cell line)
Mouse cell lines
MC3T3 (embryonic calvarium)

Rat tumor cell lines

GH3 (pituitary tumor)
PC12 (pheochromocytoma)

Plant cell lines

Tobacco BY-2 cells (kept as cell suspension culture, they are model system of plant cell)

Other species cell lines

Dog MDCK kidney epithelial
Xenopus A6 kidney epithelial
Zebrafish AB9

Valuable Products From Cell Culture

From cultured animal cells several valuable products such as human monoclonal antibodies, and biochemicals can be produced on a large scale. Several million dollars have been earned from this industry in Europe, America, Africa, Japan and India. This industry has better future. More interestingly, the genetically engineered cells have revolutionized the cell culture industry. Several specific promoters of human origin are utilized for high expression of foreign genes.

For large scale production of certain biochemicals, the genetically engineered baculovirus-infected animal cells are also in use in a bioreactor. To fulfil the process several 'perfusion systems1 have been developed that retain the cells in .the bioreactor at the time of replacement of conditioned medium with fresh medium. This results in increase in cell density and in turn cell productivity. For commercial production of products a large scale cell culture system and scaling up of process are required. Therefore, 'master cell banks' (MCBs) are established to meet out the demand. The MCBs are used to develop master working cell bank (MWCB) which meets the demand of production system. After several subculturing, the MWCB is regularly checked for any kind of changes occurring in cells. Thus, the large scale cultures are the source of all valuable products which are produced in a bioreactor.

Some of the important products which are produced from animal cell cultures are : (i) enzymes (asperagenase, collagenase, urokinase, pepsin, hyaluronidase, rennin, trypsm, tyrosin hydroxylase), (ii) hormones (leutinizing hormone, follicle stimulating hormone, chorionic

hormone and erythropoietin), (iii) vaccines (foot and mouth disease vaccine, vaccines for influenza, measles and mumps, rubella and rabies), (iv) monoclonal antibodies, (v) interferons, etc. Tolbert *et al*. (1982) got success in producing large quantities of human interleukin-2 or T-cell growth factor by culturing a permanent lymphoblastoid T-cell line in a large batch suspension culture in a bioreactor.

Table: Some products of medical use derived from animal cell cultures.

Products	***Application***
Erythropoietins	
Erythropoietin-a	Anaemia resulting from cancer and chemothcraoy
Erythropoietin-p	Anaemia secondary to kindney disease
Human growth hormones	
hGH	Human growth deficiency in children, renal cell carcinoma
Somatotropin	Chronic renal insufficiency, Turners' syndrom
Monoclonal antibodies (therapeutic)	
Anti-lipopolysacharide	Treatment of sepsis
Murine anti-idiotype/human	
B-cell lymphoma	B-cell lymphoma
Monoclonal antibodies (diagnostics)	
Anti-fibrin 99	Blood clot
99 Tcm-FAb (breast)	Blood cancer
PR-356CYT-356-in-lll	Prostate adinocarcinoma
Plasminogen activator	
Urokinase type plasminogen activator	Acute myocardial infarction, acute stroke, pulmonary embolism, deep vein thrombosis
Tissue type plasminogen activator	
Recombinant plasminogen activator	
Vaccines	
HIV vaccines (gpl20)	AIDS prophylaxis and treatment
Malaria vaccine	Malaria prophylaxis
Polio vaccines	Poliomyelitis prophylaxis

Recombinant proteins from cell cultures:

Interferons

Interferons (IFNs) are a group of signaling proteins made and released by host cells in response to the presence of several pathogens, such as viruses, bacteria, parasites, and also tumor cells. In a typical scenario, a virus-infected cell will release interferons causing nearby cells to heighten their anti-viral defenses.

IFNs belong to the large class of proteins known as cytokines, molecules used for communication between cells to trigger the protective defenses of the immune system that help eradicate pathogens. Interferons are named for their ability to "interfere" with viral replication by protecting cells from virus infections. IFNs also have various other functions: they activate immune cells, such as natural killer cells and macrophages; they increase host defenses by up-regulating antigen presentation by virtue of increasing the expression of major histocompatibility complex (MHC) antigens. Certain symptoms of infections, such as fever, muscle pain and "flu-like symptoms", are also caused by the production of IFNs and other cytokines.

More than twenty distinct IFN genes and proteins have been identified in animals, including humans. They are typically divided among three classes: Type I IFN, Type II IFN, and Type III IFN. IFNs belonging to all three classes are important for fighting viral infections and for the regulation of the immune system.

Types of interferon

Based on the type of receptor through which they signal, human interferons have been classified into three major types.

Interferon type I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α/β receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The type I interferons present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. In general, type I interferons are produced when the body recognizes a virus has invaded it. They are produced by fibroblasts and monocytes. However, the production of type I IFN-α is prohibited by another cytokine known as Interleukin-10. Once released, type I interferons will activate molecules which prevent the virus from producing and replicating its RNA and DNA. Overall, IFN-α can be used to treat hepatitis B and C infections, while IFN-β can be used to treat multiple sclerosis.

Interferon type II (IFN-γ in humans): This is also known as immune interferon and is activated by Interleukin-12. Furthermore, type II interferons are released by T helper cells, type 1 specifically. However, they block the proliferation of T helper cells type two. The previous results in an inhibition of Th2 immune response and a further induction of Th1 immune response, which leads to the development of debilitating diseases such as multiple sclerosis. IFN type II binds to IFNGR, which consists of IFNGR1 and IFNGR2 chains and has a different receptor than type I IFN.

Interferon type III: Signal through a receptor complex consisting of IL10R2 (also called CRF2-4) and IFNLR1 (also called CRF2-12). Although discovered more recently than type I and type II IFNs, recent information demonstrates the importance of Type III IFNs in some types of virus infections.

In general, type I and II interferons are responsible for regulating and activating the immune response. Expression of type I and III IFNs can be induced in virtually all cell types upon recognition of viral components, especially nucleic acids, by cytoplasmic and endosomal receptors, whereas type II interferon is induced by cytokines such as IL-12, and its expression is restricted to immune cells such as T cells and NK cells.

Viral vaccines

Gene therapy

There are many diseases which can be cured by using specific medicine synthesized biochemically. Now-a-days techniques have been developed to produce recombinant therapeutic biochemicals, for example, insulin, interferon, somatotropin, somatostatin, endorphin, human blood clotting factor VIII:C, immunogenic proteins, etc. Several companies viz., Eberstadt & Co. (New York), E. Lily (USA), National Pituitary Agency (USA), Kabi Vitrum AB (Sweden), Genetech Co (USA), Biogen (Switzerland), Hybritech (USA), Astra Research Center (India), etc. are producing or trying to produce on mass scale to make available at low cost.

However, after 1975, a remarkable advancement in recombinant DNA technology has occurred and accumulated such knowledge that has made possible to transfer genes for treatment of human diseases. Several protocols have been developed for the introduction and expression of genes in humans, but the clinical efficiency has to be demonstrated conclusively. Success of gene therapy depends on the development of better gene transfer vectors for sustained, long term expression of foreign gene as well as a better understanding of gene physiology of human diseases (Rangarajan and Padmanaban, 1996).

Genes are the ultimate molecular switches that control various cellular process. The abnormal gene expression can manifest in the form of specific genetic disorders. Until the last decade, delivering genes into humans to correct diseases has been accepted as scientifically viable and recognized as an independent discipline and christened 'gene therapy'.

The ultimate goal of gene therapy is the gene replacement therapy. Gene replacement therapy permits physiological regulation of the transgenes and elimination of the possibility of insertional activation of other cellular genes which occur at the time of random integration of the foreign gene. At present the current strategy for gene therapy largely centers around gene augmentation therapy, where the foreign gene replaces the detective or missing gene.

Overall, there are two gene transfer strategies: (i) the in vivo approach which involves introduction of genes directly into the target organs of an individual (it is done in patients therefore, also called patient therapy), (ii) ex vivo approach where cells are isolated for gene transfer in vitro followed by transplantation of genetically modified cells back into the patients (Verma. 1990).

Types of gene therapy

All the gene therapies that can be done in humans can be classified into the following four types :

(i) Somatic gene therapy. The genetic defects are corrected in somatic cells of the body. It was initially formulated for the treatment of monogenetic defects, but now holds promises for a wide range of disorders such as cancer, neurological disorders, heart diseases and infectious diseases

(Table). Sufficient expertise in performing successful gene transfer in somatic cells is required before carrying out gene manipulation in humans (Anderson, 1992).

(ii) Germ-line gene therapy. The functional genes are introduced into the germ cells for correction of genetic defects in the offspring. This therapy is being carried out in laboratory and farm animals. However, it has not been attempted in humans due to technical and ethical problems. One of its types is the embryo therapy where embryos are diagnosed for genetic defects. If any such disease is present the patients are advised for embryo therapy or abortion. In young embryo a functional gene is transferred through microinjection technique (Mandal, 1988).

(iii) Enhancement genetic engineering. This type of gene transfer is done for the improvement of a specific trait in animals; for example introduction of growth hormone gene to increase height. It is being carried out in laboratory and farm animals.

Table: Genetic disorders and acquired diseases amenable to gene therapy.

Diseases	*Therapeutic agent*	*Strategy*	*Vector*	*Target cell/tissue*
Genetic Disorders				
Cystic fibrosis	CFTR	*In vivo*	Adenovirus	Nasal epithelium
Familial hyper-	LDL	*In vivo*	Cationic lipid	Nasal epithelium
cholesterolaemia				
SCID	ADS	*Ex vivo*	Retrovirus	T cells
Haemophilia	Factor VIII/IX	*In vivo*	Retro virus	Hepatocytes, skin, muscles
DMD	Dystrophin	*In vivo*	Retrovirus	Skeletal muscles
		Ex vivo	Retrovirus	Myoblasts
Acquired Disorders				
Alzheimer's disease	NGF	*Ex vivo*	Retrovirus	Tumour cells
AIDS	HIV antigen	*Ex vivo*	Retrovirus	T cells
	RevMlO	*Ex vivo*	Retrovirus	Hepatocyte
	Cytokine	*Ex vivo*	Retrovirus	Hematopoietic stem cells
Cancer	Interleukins,	*Ex vivo*	Retrovirus	Tumour cells
	HSV-TK	*Ex vivo*	Retrovirus	Tumour cells
	HLA-B4	*In vivo*	Catonic lipid	Tumour cells
	Tumour suppressor	*In vivo*	Catonic lipid	Tumour cells
	Gene			
Cardiovascular	tPA	*In vivo*	Adenovirus	Tumour cells

Disease				
Parkinson's Disease	TH	*Ex vivo*	Retrovirus	Fibroblast

CFTR, cystis fibrosis transmembrane regulator; SCID, severe combined immunodeficiency syndrome; DMD, Duchemme muscular systrophy, ADA, adenosine deaminase; HSV-TK, herpes simplex virus thymidine virus, NGF, nerve growth factor TH, tyrosine hydroxylase,tPA, tissue plasminogenactivator.

(iv) Eugenic genetic engineering. Novel genes can be introduced in humans to alter or improve complex traits such as intelligence and personality. This type of therapy is not being attempted in humans because it is far beyond our technical capabilities, and ethical problems.

In 1990, for the first time, Michaele Blease and W. French Andresco of National Institute of Health, Bethesda, U.S.A. attempted gene therapy on a human patients. A four year old girl was suffering from 'severe combined immunodeficiency' (SCID). This disease is caused by a faulty gene which expresses the enzymes adenosine deaminase (ADA). Deficiency of ADA results in the production of a chemical which selectively destroys the T- and (3-cells of the immune systems. Finally, the patients die. The scientists introduced a healthy ADA gene into the body of the girl who protected her immune system from damage. This successful trial has given the signal for the dawn of a new era in the field of medical sciences.

POSSIBLE QUESTIONS

8 MARKS

1. Explain in detail on artificial insemination and embryo transfer.
2. Discuss in detail on gene therapy.
3. Explain in detail on applications of transgenic animals.
4. Discuss on primary cell culture techniques.
5. What you know about *In vitro* fertilization? Explain in detail.
6. Elaborate in detail about animal cell culture.
7. Discuss about production of transgenic animals and its applications.
8. Explain in detail about recombinant proteins from cell cultures.

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