



3 Main Classification of Vectors (With Diagram)

Article shared by : **Priyanka Parmar**

The classifications are: 1. On the Basis of Our Aim with Gene of Interest 2. On the Basis of Host Cell Used 3. On the Basis of Cellular Nature of Host Cell.

Vector Classification # 1.

On the Basis of Our Aim with Gene of Interest:

The point is what we are targeting from our gene of interest – its multiple copies or its protein product.

Depending on these criteria vectors are of following two types:

1. Cloning Vectors:

We use a cloning vector when our aim is to just obtain numerous copies (clones) of our gene of interest (hence the name cloning vectors). These are mostly used in construction of gene libraries. A number of organisms can be used as sources for cloning vectors.

Some are created synthetically, as in the case of yeast artificial chromosomes and bacterial artificial chromosomes, while others are taken from bacteria and bacteriophages. In all cases, the vector needs to be genetically modified in order to accommodate the foreign DNA by creating an insertion site where the new DNA will fitted. Example: PUC cloning vectors, pBR322 cloning vectors, etc.

2. Expression Vectors or Expression construct:

We use an expression vector when our aim is to obtain the protein product of our gene of interest. To get the protein we need to allow the expression of our gene of interest (hence the name expression vector) by employing the processes of transcription and translation.

Apart from the three DNA sequences discussed above (origin of replication, selectable markers and multiple cloning sites), the expression vectors have some special additional sequences as well.

Those are as follows:

- a. A bacterial promoter, such as the lac promoter. The promoter precedes a restriction site where foreign DNA is to be inserted, allowing transcription of foreign sequence to be regulated by adding substances that induce the promoter.
- b. A DNA sequence that, when transcribed into RNA, produces a prokaryotic ribosome binding site.
- c. Prokaryotic transcription initiation and termination sequences.
- d. Sequences that control transcription initiation, such as regulator genes and operators.

In some types of expression vectors which are specifically used in association with the bacterial host (like *E. coli*), multiple cloning site is not immediately

adjacent to the ribosome binding sequence, but instead is preceded by a special sequence coding for a bacterial polypeptide.

While using such type of expression vectors the gene of interest is inserted just after the gene for bacterial polypeptide. In this way we fuse two reading frames, producing a hybrid gene that starts with the bacterial gene and progresses without a break into the codons of our gene of interest.

The product of gene expression is therefore a hybrid protein, consisting of short bacterial polypeptide fused into amino terminus of our target polypeptide sequence. This hybrid polypeptide chain consisting of two different types of polypeptides is called a fusion protein.

The followings are the reasons for incorporation of a fusion protein before our gene of interest:

(a) The presence of bacterial peptide at the start of fusion protein may stabilize the molecule and prevent it from being degraded by the host cell. In contrast the foreign polypeptides that lack a bacterial segment are often destroyed.

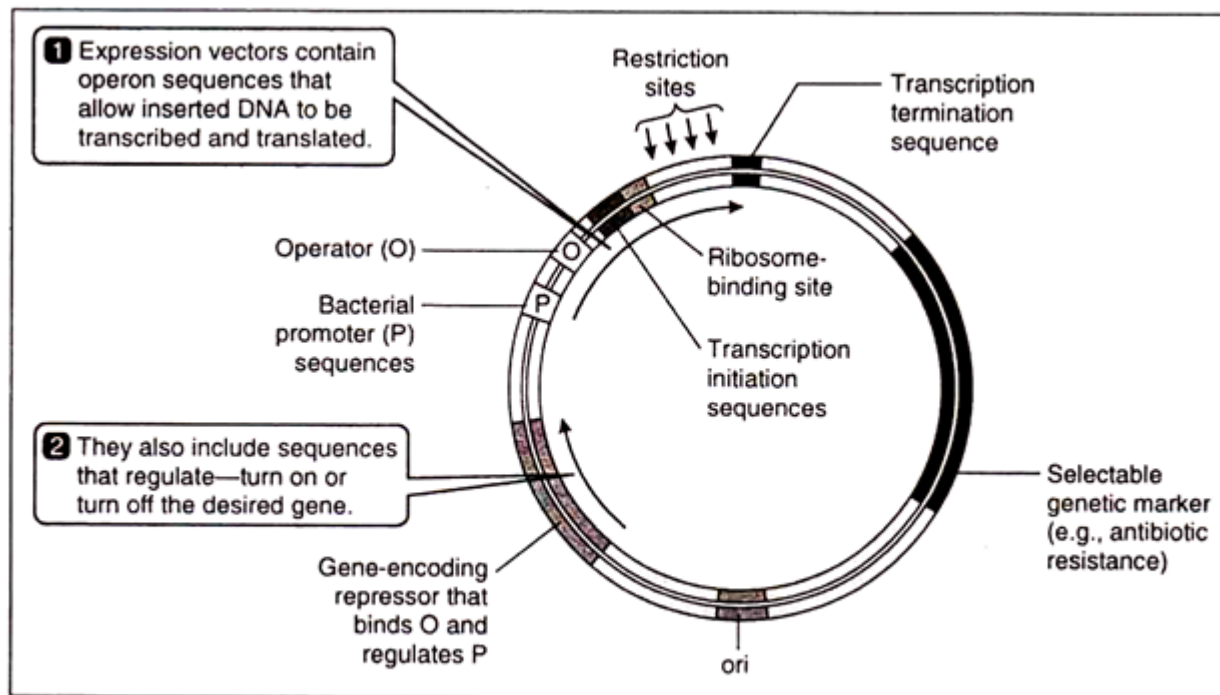


Fig. 4.11: The key features of a typical expression vector

(b) The bacterial polypeptide may act as a signal peptide, responsible for transporting our target protein to a specific location from where these are collected. For example, if the bacterial peptides are derived from a protein that is exported by the cell (e.g.; products of *ompA* genes), then our target polypeptide will simply be transported outside of the host cell straight into the culture media from where these can be collected.

(c) The bacterial polypeptide may also help in purification of the target polypeptide by different purification techniques such as affinity chromatography.

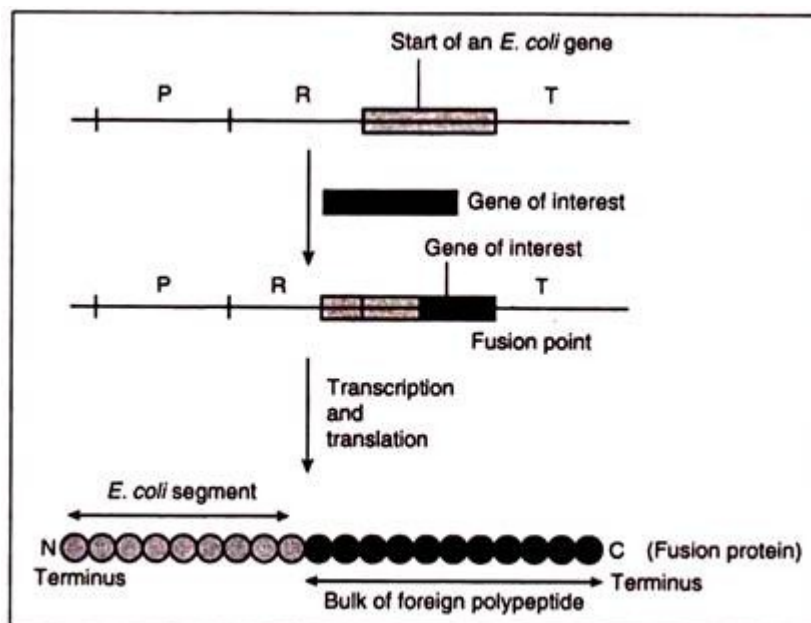


Fig. 4.12: The construction of a hybrid gene and synthesis of a fusion proteins

Vector Classification # 2.

On the Basis of Host Cell Used:

After construction of a recombinant DNA these can be introduced into a host cell. So depending on the host cell the vectors are designed and constructed. All the parts of the vectors must be functionally compatible with the host. For example, if we are making a vector for a bacterial host, it must have a suitable origin of replication which will be functional in a bacterial cell.

Depending on this basis the vectors are classified as under:

1. Vectors for Bacteria:

These are special bacterial origin of replication and antibiotic resistance selectable markers. Bacteria support different kinds of vectors, e.g.; plasmid vectors, bacteriophages vectors, cosmids, phasmids, phagemids, etc.

2. Vectors for Yeast:

They have special origin of replication called as autonomously replicating sequences (ARS), e.g., yeast replicative plasmid vectors (YRp) etc.

3. Vectors for Animals:

These vectors are needed in biotechnology for the synthesis of recombinant protein from genes that are not expressed correctly when cloned in *E. coli* or yeast, and methods for cloning in humans are being sought by clinical molecular biologists attempting to devise techniques for gene therapy, in which a disease is treated by introduction of a cloned gene into the patient, e.g., P-element, SV40 etc.

4. Vectors for Plants:

The production of genetically modified plants has become possible due to successful use of plant vectors. e.g., Ti-plasmid, Ri-plasmid etc.

Vector Classification # 3.

On the Basis of Cellular Nature of Host Cell:

On this basis, the vectors are of two types:

1. Prokaryotic Vectors:

This comprises of all vectors for bacterial cells.

2. Eukaryotic Vectors:

This comprises of all the vectors for yeast, animal and plant cells.

Prokaryotic Vectors (Bacterial Vectors):

The *E. coli* cell which is frequently used as a prokaryotic host needs specific types of vectors which are designed accordingly to function in its cytoplasm. Plasmid based and bacteriophage based vectors are most common prokaryotic vectors. The prokaryotic vectors include plasmid derived vectors,

bacteriophage derived vectors, phagemid vectors, plasmid vectors and fosmid vectors.

These are discussed as follows:

Plasmid Vectors:

These are the most common vectors for the prokaryotic host cells. Bacteria are able to express foreign genes inserted into plasmids (Fig. 4.13). Plasmids are small, circular, double-stranded DNA molecules lacking protein coat that naturally exists in the cytoplasm of many strains of bacteria.

Some of the examples of naturally occurring plasmids are Ti plasmids, F-factors, R-factors, Co/E1 plasmid, etc. Plasmids are independent of the chromosome of bacterial cell and range in size from 1000 to 200 000 base pairs. Using the enzymes and 70s ribosomes that the bacterial cell houses, DNA contained in plasmids can be replicated and expressed.

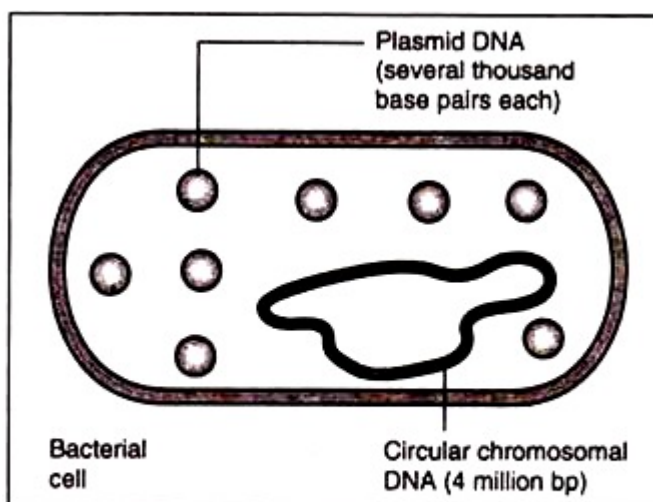


Fig. 4.13: Chromosomal and plasmid DNA coexist in an endosymbiotic relationship

The bacterial cells benefit from the presence of plasmids, which often carry genes that express proteins able to confer antibiotic resistance. These also protect bacteria by carrying genes for resistance to toxic heavy metals, such as mercury, lead, or cadmium.

In addition, some bacteria carry plasmids possessing genes that enable bacteria to break down herbicides, certain industrial chemicals, or the components of petroleum. The relationship between bacteria and plasmids is endosymbiotic; both the bacteria and plasmids benefit from mutual arrangement. Plasmids also possess characteristic copy number.

The higher the copy number, higher is the number of individual plasmids in a host bacterial cell. If more copies of plasmid exist, more protein will be synthesized because of the larger number of gene copies carried by the plasmid. The number of copies plays a role in phenotypic manifestation of a gene. For example, the more copies of an antibiotic-resistance gene there are, the higher the resistance to the antibiotic.

It is very important to note that naturally occurring plasmids do not have all necessary sequences which are required by a DNA molecule to act as a profitable vector. Due to this, natural plasmids are extracted and modified by inserting suitable DNA segments and a complete vector DNA molecule is made.

Plasmid-cloning vectors are derived from bacterial plasmids and are the most widely used, versatile, and easily manipulated ones.

The following are different types of plasmid vectors:

I. pBR322:

This was the first widely used, purpose built plasmid vector. pBR322 has a relatively small size of 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. Also this vector has a reasonably high copy number (~15 copies per cell), which can be

increased 200-fold by treatment with a protein-synthesis inhibitor—chloramphenicol amplification.

The nomenclature of pBR 322:

The nomenclature of 'pBR 322' can be understood with following explanation:

1. 'p' indicates as a plasmid
2. 'BR' identifies Bo-liver and Rodriguez, the two researchers who developed it
3. '322' distinguishes those plasmids from others (like pBR 325, pBR 327, etc.) developed in the same laboratory.

The Construction of pBR322:

1. Origin of Replication:

It carries a fragment of plasmid pMB1 that acts as an origin for DNA replication and thus ensures multiplication of the vector.

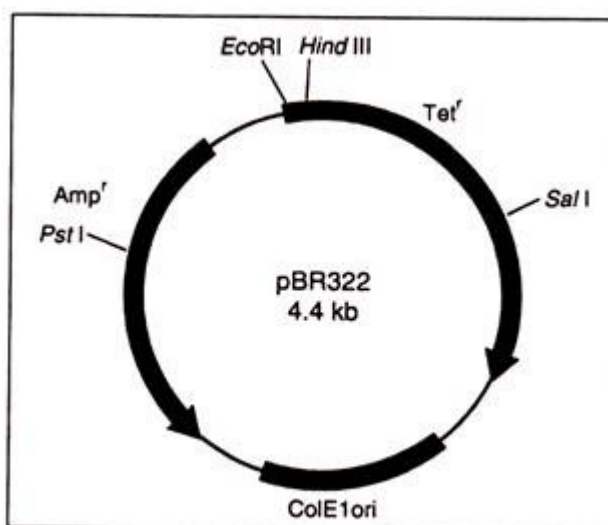


Fig. 4.14: Diagram sketch of pBR322

2. Selectable Marker:

It carries two antibiotic resistance genes—ampicillin and tetracycline.

3. 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 BamHI 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.

Pedigree of pBR322:

By pedigree we understand the origin of pBR322. pBR322 is not a naturally occurring plasmid. It is manufactured by following certain steps which are outlined in (Fig. 4.15). It is important to note that pBR322 comprises DNA derived from three different naturally occurring plasmids.

The amp^R gene originally resided on the plasmid R1 (a naturally occurring antibiotic resistant plasmid in *E. coli*), the tet^R is derived from R6-5 (a second antibiotic resistant plasmid) and the origin of replication is derived from pMB1, which is closely related to the Colicin producing plasmid ColE1.

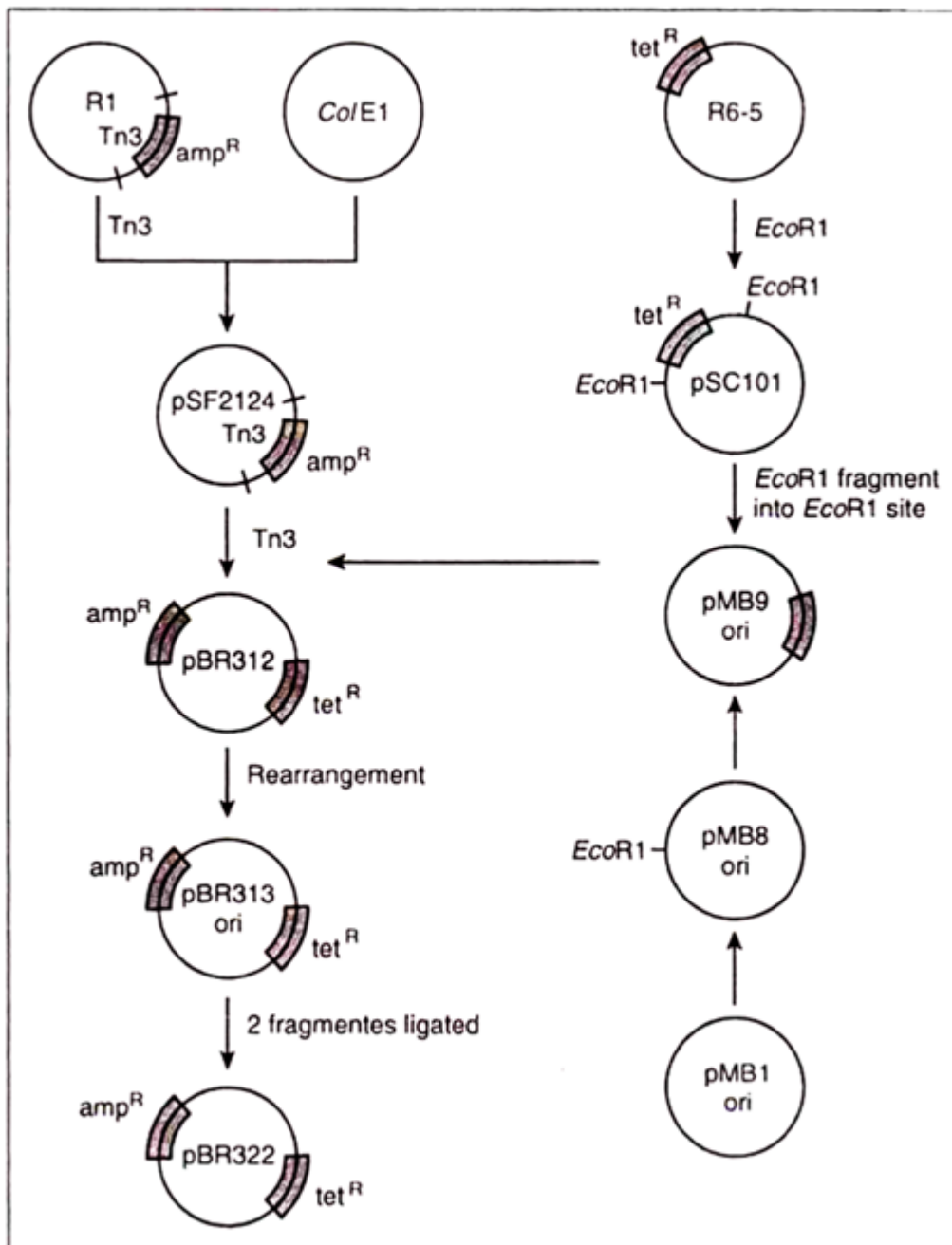


Fig. 4.15: The Pedigree of pBR322

Recombinant selection with pBR322 – in- sectional inactivation of an antibiotic resistance gene.

When we have introduced our recombinant DNA (vector + gene of interest) into the host cell (by a process called transformation and the host cells that takes up the recombinant DNA are called transformed host cells) then we have to screen the entire host population in order to select the transformed cells

(with recombinant DNA) from the non-transformed one (without recombinant DNA).

Every vector has some mechanism associated with it for this screening.

Here we will discuss what is the mechanism followed by the pBR322 vector in this regard. pBR322 has several unique restriction sites that can be used to open up the vector before insertion of a new DNA fragment. BamHI, for example, cuts pBR322 at just one position, within the cluster of genes that code for resistance to tetracycline.

A recombinant pBR322 molecule, one that carries an extra piece of DNA in the BamHI site is no longer able to confer tetracycline resistance on its host, as one of the necessary genes is now disrupted by the inserted DNA. Cells containing this recombinant pBR322 molecule are still resistant to ampicillin, but sensitive to tetracycline ($\text{amp}^R \text{ tet}^S$).

Screening for pBR322 recombinants is performed in the following way. After transformation the cells are plated onto ampicillin medium and incubated until colonies appear [Fig. 4.16(a)].

All of these colonies are transformants (remember, untransformed cells are amp^S and so do not produce colonies on the selective medium), but only a few contain recombinant pBR322 molecules: most contain the normal, self-ligated plasmid. To identify the recombinants the colonies are replica plated onto agar medium that contains tetracycline [Fig. 4.16(b)].

After incubation, some of the original colonies regrow, but others do not [Fig. 4.16(c)]. Those that do grow consist of cells that carry the normal pBR322 with no inserted DNA and, therefore, a functional tetracycline resistance gene cluster ($\text{amp}^R \text{ tet}^R$).

The colonies that do not grow on tetracycline agar are recombinants ($amp^R tet^S$); once their positions are known, samples for further study can be recovered from the original ampicillin agar plate.

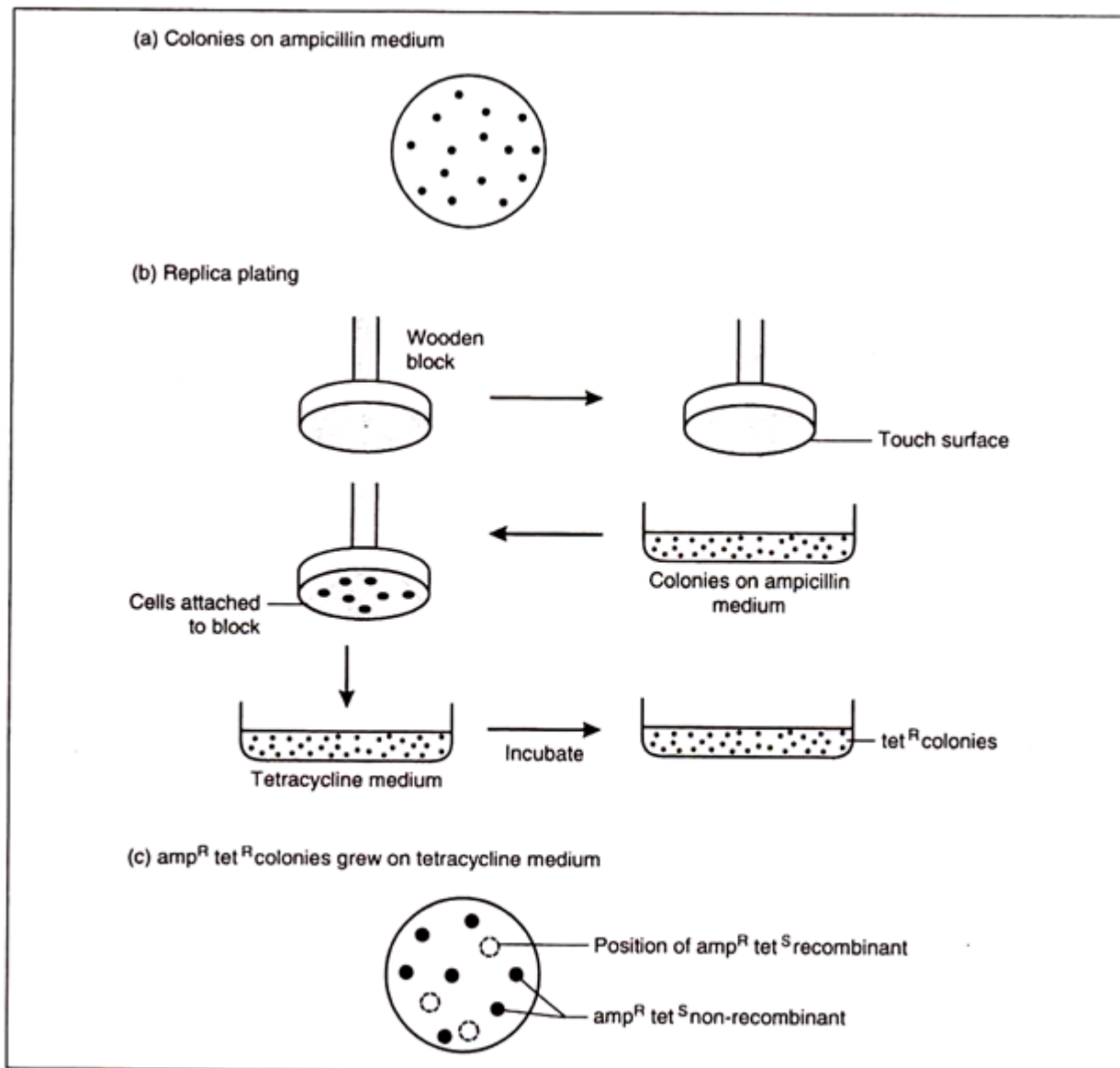


Fig. 4.16: Screening for pBR322 recombinants by insertional inactivation of the tetracycline resistance gene. (a) Cells are plated onto ampicillin agar: all the transformants produce colonies. (b) The colonies are replica plated onto tetracycline medium. (c) The colonies that grow on tetracycline medium are $amp^R tet^R$ and, therefore, non-recombinants. Recombinants ($amp^R tet^S$) do not grow, but their position on the ampicillin plate is now known

Uses of pBR322:

It is widely used as a cloning vector. In addition to this, it has been widely used as a model system for study of prokaryotic transcription and translation.

Advantages of pBR322:

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

Disadvantages of pBR322:

1. It has very high mobility i. e; it can move to another cell in the presence of a conjugative plasmid like F-factor. The *nic-bom* (*bom*=basis of mobility) region of pBR322 is responsible for this feature. Due to this, the vector may get lost in a population of mixed host cells.
2. There is a limitation in the size of the gene of interest that it can accommodate.
3. Not a very high copy number is present as is expected from a good vector.
4. Although insertional inactivation of an antibiotic resistance gene provides an effective means of recombinant identification, the method is made inconvenient by the need to carry out two screenings, one with the antibiotic that selects for trans-formants, followed by the second screen, after replica plating, with the antibiotic which distinguishes recombinants.

This makes the screening process time- consuming and laborious.

Another vector pBR327 was derived from pBR322, by deletion of nucleotides between 1,427 to 2,516. These nucleotides are deleted to reduce the size of the vector and to eliminate sequences that were known to interfere with the expression of cloned DNA in eukaryotic cells. pBR327 still contains genes for resistance against two antibiotics (tetracycline and ampicillin).

pBR327 has following two advantages over pBR322:

1. pBR327 has high copy number (30-45 copies per cell).
2. It lacks mobility.

II. pUC Vectors:

pUC are obtained by modifying the pBR322 vector. pUC vectors are smaller than pBR322 of being only ~2.7 kb. But comparatively they have a high copy number. A mutation within the origin of replication produces 500 to 600 copies of the plasmid per cell without amplification.

The Nomenclature of pUC Vectors:

The nomenclature of 'pUC' can be understood with the following explanation:

1. 'p' indicates the plasmid.
2. 'UC' stands for university of California where it was first developed by J. Messing et al.

We also see many numbers after this like pUC8, pUC18, pUC19 and so on. They are just the series of pUC and have been named just to separate from each other.

The construction of pUC vectors:

1. **Origin of Replication:** It is derived from the origin of replication of pBR322. The ColE1 origin of replication of pBR322 has been modified by carrying out a chance mutation so that each transformed E. coli cell has 500-600 copies of the plasmid.

2. Selectable Marker: It has an ampicillin resistant gene. The transformed host cells can grow on media having ampicillin whereas non-transformed cells die.
3. lac Z' gene having MCS.
4. The lac Z' is incorporated into this vector codes for the enzyme beta-galactosidase which acts on a chromogenic substrate called X-gal (present in bacterial culture media). The expression of lac Z' gene is induced by another compound present in the same media called Iso-propyl-thiogalactoside (IPTG).

When the enzyme substrate reaction takes place, then the X-gal is converted from white to a blue compound. Now the lac Z' gene itself has MCS. Hence, when the gene of interest has been introduced into the lac Z' gene, then it fails to code for beta-galactosidase and thus in this case the substrate (X-gal) is never converted to any other colour. This type of screening is called blue-white screening.

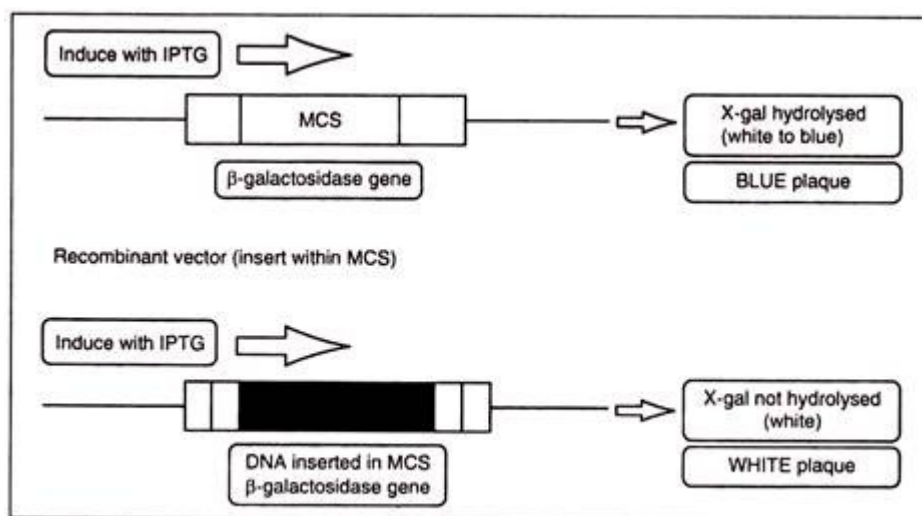


Fig. 4.17: Principle of blue-white selection for the detection of recombinant vectors

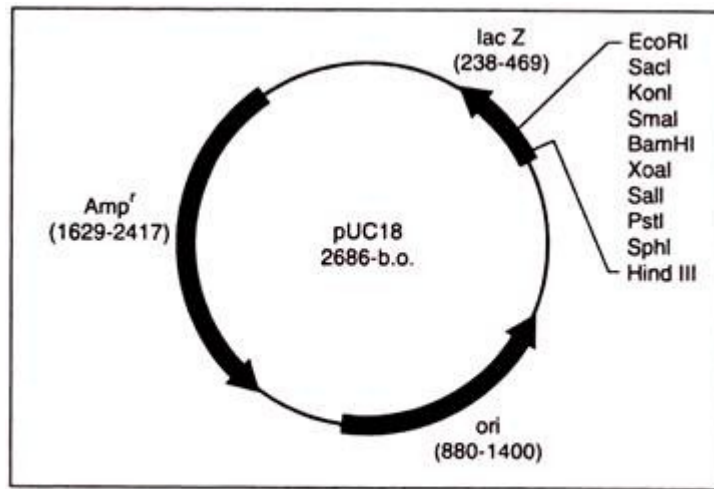


Fig. 4.18: Diagram sketch of pUC18. It differs from the pUC19 only in the region of polylinker site in the arrangement of restriction sites

Pedigree of pUC Vectors:

During the construction of pUC the only selectable marker that is kept out of pBR322 is ampicillin resistant gene. But all the MCS are removed from the amp^R by carrying out chance mutations. The ColE1 origin of replication is also modified by the same process so that it can smoothly carry out the process of replication again and again ultimately increasing the copy number of the vector.

Along with this a lac Z' sequence coding for beta galactosidase is also inserted. Similarly after this by the process of chance mutation we create MCS within the lac Z' sequence (Fig. 4.19).

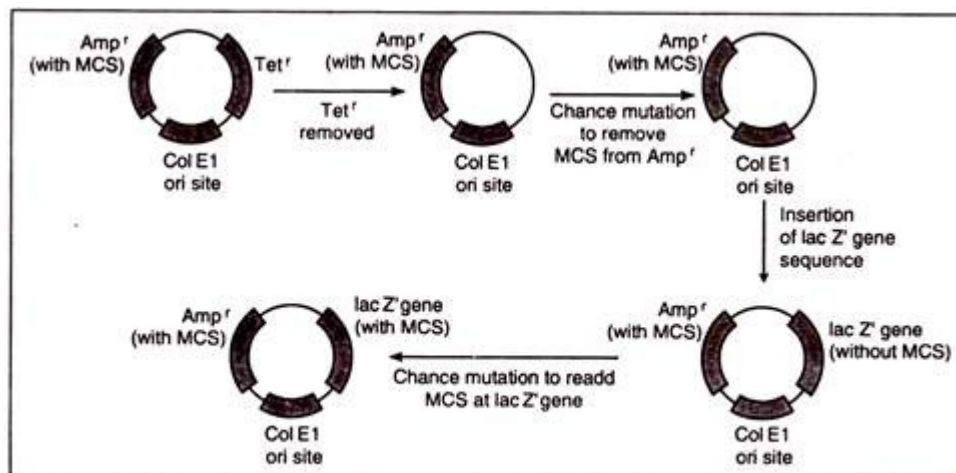


Fig. 4.19: Pedigree of pUC

Screening of Transformed Host Cells using pUC Vectors:

After transforming the host cells we carry out their screening to select the transformed cells from non-transformed ones. pUC8 [Fig. 4.20(a)], which carries the ampicillin resistance gene and a gene called lac Z', which codes for part of the enzyme beta-galactosidase.

Cloning with pUC8 involves insertional inactivation of the lac Z' gene, with recombinants identified because of their inability to synthesize beta-galactosidase [Fig. 4.20(b)].

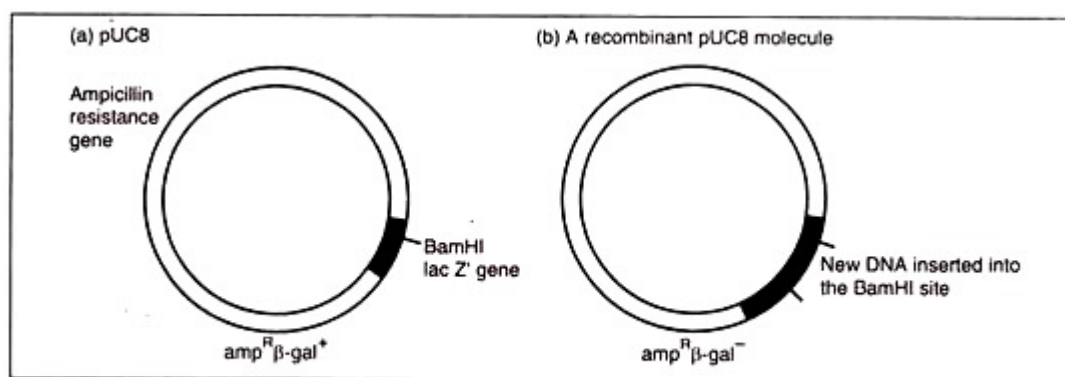


Fig. 4.20: The cloning vector pUC8: (a) the normal vector molecule; (b) a recombinant pUC8 molecule containing an extra piece of DNA inserted into the BamHI site

Beta-Galactosidase is one of the series of enzymes involved in breakdown of lactose to glucose plus galactose. It is normally coded by the gene lac Z, which resides on E. coli chromosome. Some strains of E. coli have a modified lac Z gene, one that lacks the segment referred to as lac Z' and coding for the a-peptide portion of beta-galactosidase [Fig. 4.21(a)].

These mutants can synthesize the enzyme only when they harbour a plasmid, such as pUC8, that carries the missing lac Z' segment of the gene.

A cloning experiment with pUC8 involves selection of trans-formants on ampicillin agar followed by screening for beta-galactosidase activity to identify recombinants. Cells that harbour a normal pUC plasmid are amp^R and able to

synthesize beta-galactosidase [Fig. 4.21(a)]; recombinants are also amp^R but unable to make beta-galactosidase [Fig. 4.21(b)].

Screening for beta-galactosidase presence or absence is, in fact, quite easy. Rather than assay for lactose being split to glucose and galactose, we test for a slightly different reaction that is also catalysed by beta-galactosidase.

This involves a lactose analogue called X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) which is broken down by beta-galactosidase to a product that is coloured deep blue.

If X-gal (plus an inducer of the enzyme such as Iso-pro-pylthiogalactoside, IPTG) is added to the agar, along with ampicillin, then non-recombinant colonies, the cells of which synthesize beta-galactosidase, will be coloured blue, whereas recombinants with a disrupted lac Z' gene and unable to make p-galactosidase, will be white.

This system, which is called Lac selection, is summarized in [Fig. 4.21(b)]. Note that both ampicillin resistance and the presence or absence of p-galactosidase is tested on a single agar plate. The two screenings are, therefore, carried out together and there is no need for the time-consuming replica-plating step that is necessary with plasmids such as pBR322.

Vectors	Features	Applications	Supplier
pBR322	Ap ^r Tc ^r Single cloning sites	General cloning and subcloning in <i>E. coli</i>	Various
pAT153	Ap ^r Tc ^r Single cloning sites	General cloning and subcloning in <i>E. coli</i>	Various
pGEM [®] -3Z	Ap ^r MCS SP6/T7 promoters <i>lac Z</i> α-peptide	General cloning and <i>in vitro</i> transcription in <i>E. coli</i> and single-stranded DNA production	Promega
pCf [®]	Ap ^r MCS T7 promoter CMV enhancer/promoter	Expression of genes in mammalian cells	Promega
pET-3	Ap ^r MCS T7 promoter	Expression of genes in bacterial cells	Stratagene
pCMV-Script [®]	Neo ^r Large MCS CMV enhancer/promoter	High level expression of genes in mammalian cells and cloning of PCR products	Stratagene

Uses of pUC Vectors:

pUC vectors can be used both as cloning vector and expression vector. When used as an expression vector its sequences are slightly modified to meet necessary requirements.

Advantages of pUC Vectors:

The pUC vectors offer following major advantages over pBR322 vectors:

- (a) High copy number of 500-600 copies per cell.
- (b) Easy and single step selection.
- (c) The unique restriction sites used for cloning are clustered within the MCS. This allows cloning of a DNA fragment having two different sticky ends.

Disadvantages of pUC:

It cannot accommodate a gene of interest larger than 15kb.

Fosmid Vectors:

These are similar to cosmids but are based on the bacterial F-plasmid. The cloning vector is limited, as a host (usually *E. coli*) can only contain one fosmid molecule. Fosmids are 40 kb of random genomic DNA. Fosmid library is prepared from a genome of target organism and cloned into a fosmid vector.

Low copy number offers higher stability than comparable high copy number cosmids. Fosmid system may be useful for constructing stable libraries from complex genomes.

Bacteriophage Derived Vectors:

Bacteriophages, or phages as they are commonly known, are viruses that specifically infect bacteria. Like all viruses, phages are very simple in structure, consisting merely of a DNA (or occasionally ribonucleic acid (RNA)) molecule carrying a number of genes, including several for replication of the phage, surrounded by protective coat or capsid made up of protein molecules (Fig. 4.22).

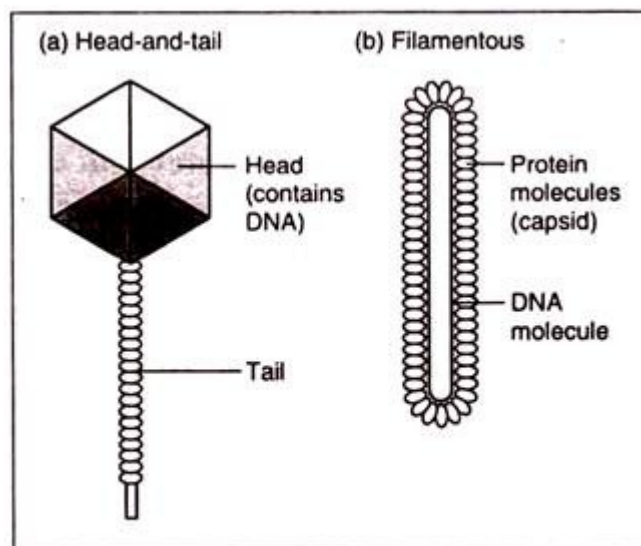


Fig. 4.22: Two main types of phage structure: (a) head-and-tail (e.g., Lamda phage); (b) filamentous (e.g., M13)

The general pattern of infection, which is the same for all types of phage, is a three-step process:

1. The phage particle attaches to the outside of bacterium and injects its DNA chromosome into the cell.
2. The phage DNA molecule is replicated, usually by specific phage enzymes coded by genes on the phage chromosome.
3. Other phage genes direct synthesis of protein components of capsid, and new phage particles are assembled and released from the bacterium.

With some phage types the entire infection cycle is completed very quickly, possibly in less than 20min. This type of rapid infection is called a lytic cycle, as release of the new phage particles is associated with lysis of the bacterial cell.

The characteristic feature of a lytic infection cycle is that phage DNA replication is immediately followed by synthesis of capsid proteins, and the phage DNA molecule is never maintained in a stable condition in the host cell. In contrast to a lytic cycle, lysogenic infection is characterized by retention of the phage DNA molecule in the host bacterium, possibly for many thousands of cell divisions.

Fred Blatter and his colleagues were the first to develop a bacteriophage as vector.

The following are different types of plasmid vectors:

I. Bacteriophage M13 vectors:

General Biology:

The M13 family of vectors is derived from bacteriophage M13. This is a male specific (infects *E. coli* having *f. pili*), lysogenic filamentous phage with a circular single-stranded DNA genome about 6,407 bp (6.4 kb) in length. Once inside the host-cell the single-stranded DNA of M13 phage acts as the template

for synthesis of a complementary strand, resulting in normal double-stranded DNA [Fig. 4.23(a)].

This molecule is not inserted into the bacterial genome, but instead replicates until over 100 copies are present in the cell [Fig. 4.23(b)]. When the bacterium divides, each daughter cell receives copies of the phage genome, which continues to replicate, thereby maintaining its overall numbers per cell.

As shown in [Fig 4.23(c)], new phage particles are continuously assembled and released, about 1000 new phages being produced during each generation of an infected cell.

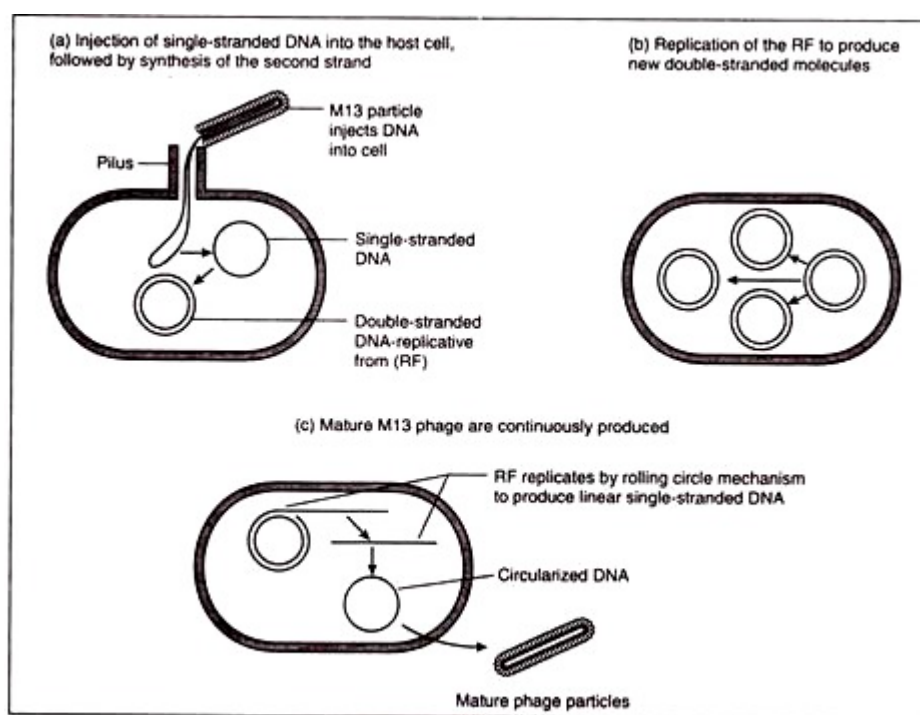


Fig. 4.23: The life cycle of M13 bacteriophage

The Attraction of M13 as a Cloning Vector:

Several features of M13 make this phage attractive as the basis for a cloning vector. The genome is less than 10 kb in size, well within the range desirable for a potential vector. In addition, the double-stranded replicative form (RF) of the M13 genome behaves very much like a plasmid, and can be treated as such for experimental purposes.

It is easily prepared from a culture of infected *E. coli* cells and can be reintroduced by transfection. Most importantly, genes cloned with an M13-based vector can be obtained in the form of single-stranded DNA. Single-stranded version of cloned genes are useful for several techniques, notably DNA sequencing and *in vitro* mutagenesis.

Using an M13 vector is an easy and reliable way of obtaining single-stranded DNA for this type of work.

Construction of M13 vectors:

The first step in the construction of M13 cloning vector is to introduce the *lac Z'* gene into the inter-genic sequence. This gives rise to M13 mp1 which forms blue plaques on X-gal agar (Fig 4.25(a)) M13 mp1 does not progress any unit restriction site in the *lac Z'* gene.

It however, contains a hexanucleotide sequence GGATTC near the start of the gene. A single nucleotide change (by using *in vitro* mutagenesis) would make this GAATTC, which is an *EcoR*1 site.

This results in the formation of M13 mp2. M13 mp2 has a slightly altered *lac Z'* gene but the beta-galactosidase enzyme produced by cells infected with M13 mp2 is still perfectly functional. M13 mp2 is the simplest M13 vector. DNA fragments with *EcoR*1 sticky ends can be inserted into the cloning site and recombinants are distinguished as clear plaques on X-gal agar.

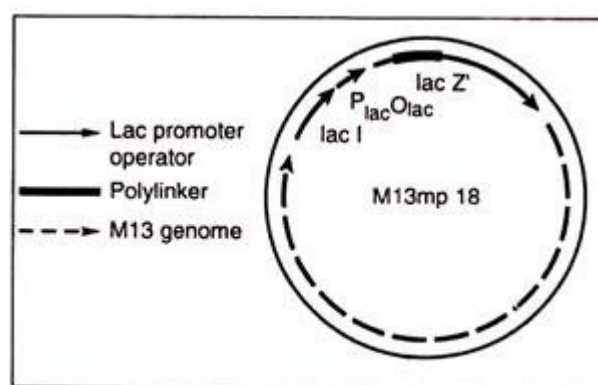


Fig. 4.24: M13 vector

We go for further modifications of M13 mp2 resulting in the production of another M13 vector called M13 mp7. In the generation of M13 mp7 first of all we synthesize a short oligonucleotide called poly-linker that consists of a series of restriction sites and has EcoR1 sticky ends. This poly-linker is inserted into the EcoRI site of M13 mp2 to generate M13mp7.

This poly-linker also provides as many as four possible cloning sites (EcoRI, BamHI, SmaI and PstI) to the new vector. It is very important to note that the poly-linker is designed so that it does not totally disrupt the lac Z' gene: a reading frame is maintained throughout the poly-linker, and a functional, though altered, beta-galactosidase enzyme is still produced.

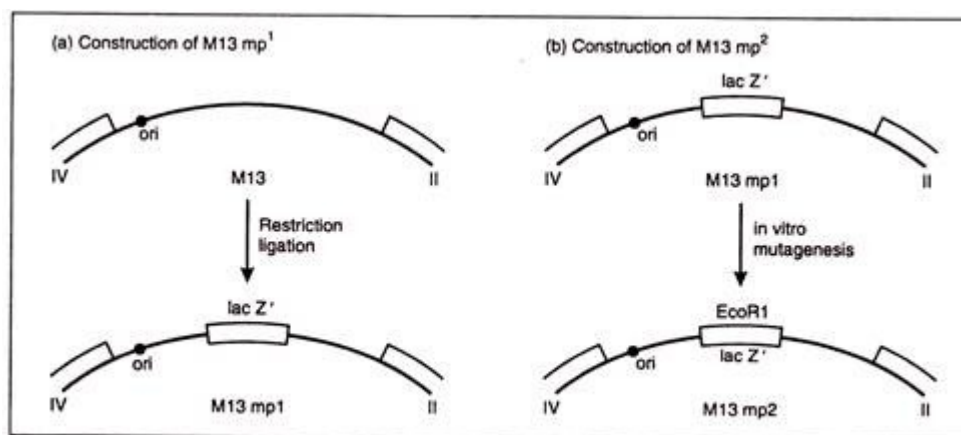


Fig. 4.25: The construction of M13mp2

Screening of transformed host cells using bacteriophage M13 vectors:

Insertion of new DNA almost invariably prevents beta-galactosidase production. So recombinant plaques are clear on X-gal agar. Alternatively, if the poly-linker is reinserted, and the original M13mp7 reformed, then blue plaques result.

Uses of bacteriophage M13 Vectors:

1. DNA Sequencing:

For a long time the most important application of M13 cloning was in DNA sequence determination by the Sanger method, also called the dideoxy or chain-termination method. This relies on synthesis of DNA in the presence of chain terminating inhibitors, the 2', 3'- di-deoxynucleoside triphosphates (ddNTPs). The method is now a very standard tool of molecular biology.

2. Phage Display Vectors:

An important use of filamentous phage is in phage display systems. Here, coding sequences are inserted into one of the coat protein genes. The result is that the phage are generated with a hybrid form of this protein, which is a fusion of the normal protein sequence and the protein product of the inserted sequence (assuming the inserted sequence has the same reading frame as the coat protein gene).

The phages are secreted from the cell, with this extra material 'displayed' on the outside. These display vectors have many uses, e.g., in screening libraries by panning and for vaccine production.

3. Other Applications:

Some protocols for site-directed mutagenesis also use single- stranded DNA, which can be obtained with vectors based on filamentous phages. Single-stranded DNA is also of particular use in generating probes for RNA analysis.

Probes can be prepared that are specific for RNA transcripts from either strand of DNA. The latter applications are outside the scope of this book, but more information can be obtained from specialized laboratory manuals.

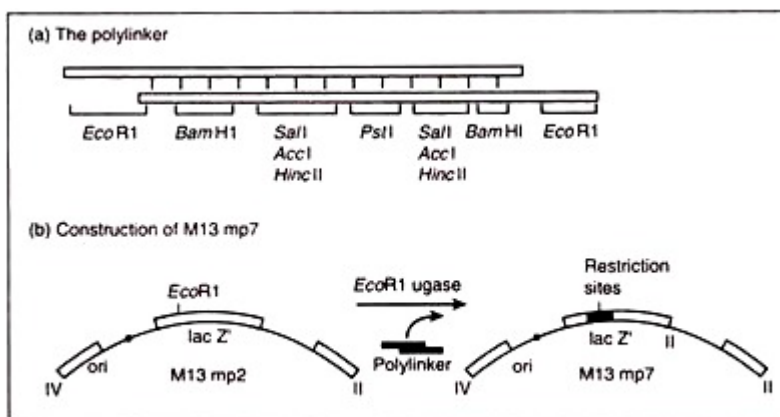


Fig. 4.26: The construction of M13mp7 from M13mp2

Advantages of bacteriophage M13 vector:

1. Advantages over Lambda Phage Vector:

M13 is an example of a filamentous phage and is completely different in structure from lambda. Furthermore, the M13 DNA molecule is much smaller than the lambda genome, being only 6407 nucleotides in length. It is circular and is unusual in that it consists entirely of single-stranded DNA.

The smaller size of the M13 DNA molecule means that it has room for fewer genes than the lambda genome. This is possible because the M13 capsid is constructed from multiple copies of just three proteins (requiring only three genes), whereas synthesis of the lambda head- and-tail structure involves over 15 different proteins.

In addition, M13 follows a simpler infection cycle than lambda, and does not need genes for insertion into the host genome. Injection of an M13 DNA molecule into an *E. coli* cell occurs via the pilus, the structure that connects two cells during sexual conjugation.

2. Other Advantages:

M13-based vectors are that they contain the same poly-linker and alpha-peptide fragments as the pUC series and recombinants can be selected by the

blue → white colour test. Also the size of the genome is below 10 kb and so is easy to handle.

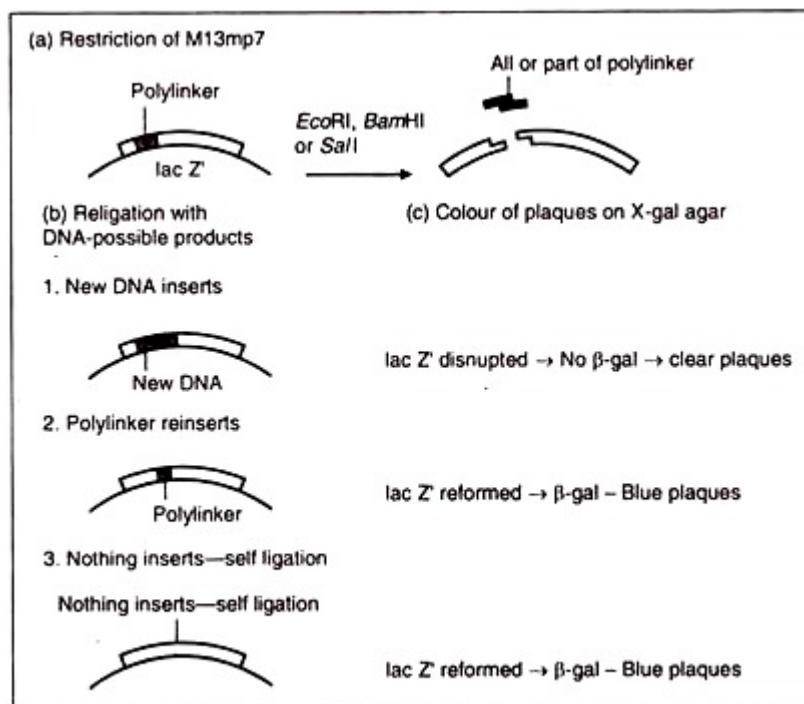


Fig. 4.27: Cloning with M13mp7

Disadvantages of bacteriophage M13 vectors:

The following are the disadvantages of bacteriophage M13 vectors:

1. Gene of interest more than 2kb cannot be cloned.
2. It has low yield of DNA.
3. The phage produce many toxins in high concentration.

II. Lamda Phage Vectors:

This is a widely used vector for the cloning of very large pieces of genes.

General Biology:

Lambda is a typical example of a head-and-tail phage. The genetic material is DNA which is present in the polyhedral head structure and the tail serves to attach the phage to the bacterial surface and to inject the DNA into the cell. The lambda DNA molecule is 49 kb in size.

It is a temperate phage and this can carry out lytic and lysogenic cycles. The positions and identities of most of the genes on the lambda DNA molecule are known (Fig. 4.28).

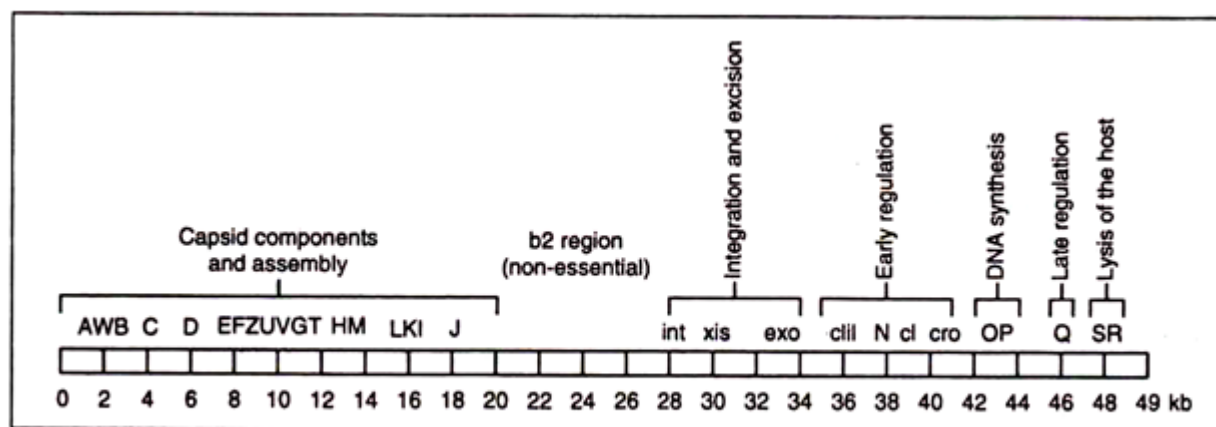


Fig. 4.28: The genetic map of lambda DNA

Lambda phage can have both linear and circular forms of DNA. The molecule shown in (Fig. 4.28) is linear, with two free ends, and represents the DNA present in the phage head. This linear molecule consists of two complementary strands of DNA, base paired according to the Watson-Crick rules. However, at either end of the molecule is a short 12-nucleotide stretch in which the DNA is single-stranded [Fig. 4.30(a)].

The two single strands are complementary, and so can base pair with one another to form a circular, completely double-stranded molecule [Fig. 4.30(b)]. Complementary single strands are often referred to as 'sticky' ends or cohesive ends, because base pairing between them can 'stick' together the two ends of a DNA molecule (or the ends of two different DNA molecules).

The lambda cohesive ends are called the cos sites and they play two distinct roles during the lambda infection cycle. First, they allow the linear DNA

molecule that is injected into the cell to be circularized, which is a necessary prerequisite for insertion into the bacterial genome (Fig. 4.29).

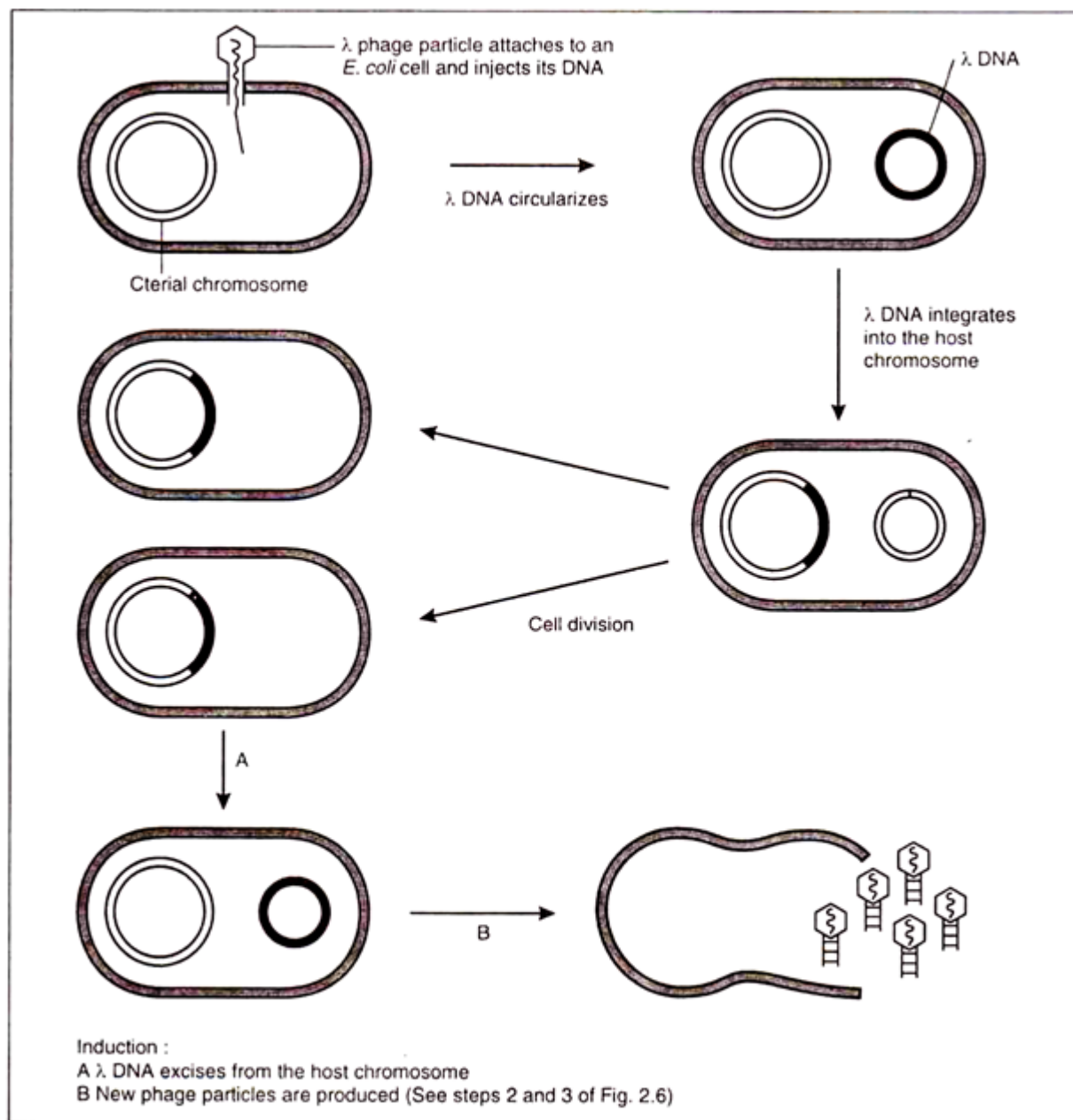


Fig. 4.29: The lysogenic cycle of lambda phage

The second role of the *cos* sites is rather different, and comes into play after the pro-phage has excised from the host genome. At this stage a large number of new lambda DNA molecules are produced by the rolling circle mechanism of replication [Fig. 4.30(c)], in which a continuous DNA strand is rolled off the template molecule. The result is a catenane consisting of a series of linear A genomes joined together at the *cos* sites.

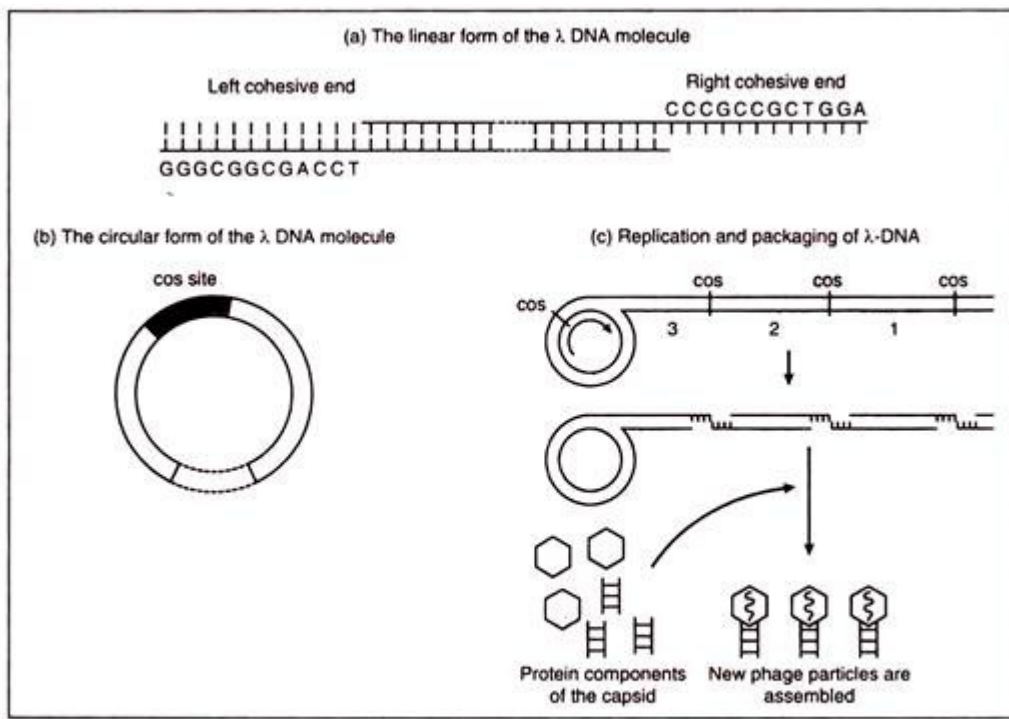


Fig. 4.30: The linear and circular forms of lambda DNA. (a) The linear form, showing the left and right cohesive ends. (b) Base pairing between the cohesive ends results in the circular form of the molecule. (c) Rolling circle replication produces a catenane of new linear lambda DNA molecules, which are individually packaged into lambda phage heads as new lambda particles are assembled

The role of the *cos* sites is now to act as recognition sequences for an endonuclease that cleaves the catenane at the *cos* sites, producing individual lambda genomes. This endonuclease, which is the product of gene A on the lambda DNA molecule, creates the single stranded sticky ends, and also acts in association with other proteins to package each lambda genome into a phage head structure.

The cleavage and packaging processes recognize just the *cos* sites and the DNA sequences to either side of them. Changing the structure of the internal regions of the lambda genome, for example, by inserting new genes has no effect on these events so long as the overall length of the A genome is not altered too greatly.

Problems associated with naturally occurring lambda phage to be used as cloning vectors:

Two problems have to be solved before lambda- based cloning vectors could be developed:

1. The lambda molecule can be increased in size by only about 5%, representing the addition of only 3kb of new DNA. If the total size of the molecule is more than 52kb, then it cannot be packaged into the lambda head structure and infective phage particles are not formed. This severely limits the size of a DNA fragment that can be inserted into an unmodified lambda vector.

2. The lambda genome is so large that it has more than one recognition sequence for virtually every restriction endonucleases. Restriction cannot be used to cleave the normal lambda molecule in a way that will allow insertion of new DNA, because the molecules would be cut into several small fragments that would be very unlikely to reform a viable lambda genome on relegation.

Due to these reasons the DNA of naturally occurring lambda phage cannot be used as a cloning vector. To solve this issue we modify the lambda's genome and make it suitable to be a successful vector.

Solving the Problems:

Solving Problem (1):

From research it has been found out that large segment in the central region of the lambda DNA molecule can be removed without affecting the ability of the phage to infect E. coli cells. Removal of this nonessential region between positions 20 and 35 on the map decreases the size of the lambda genome by up to 15kb.

This makes a room for as much as 18kb of new DNA which can be added to it to form a recombinant molecule.

This non-essential genes thus removed are involved in integration and excision of the lambda pro-phage from the E. coli chromosome. A deleted lambda genome is, therefore, non-lysogenic and can follow only the lytic infection cycle. This in itself is desirable for a cloning vector as it means that we can get

plaques (a visible structure formed within a cell culture, such as bacterial cultures within some nutrient medium).

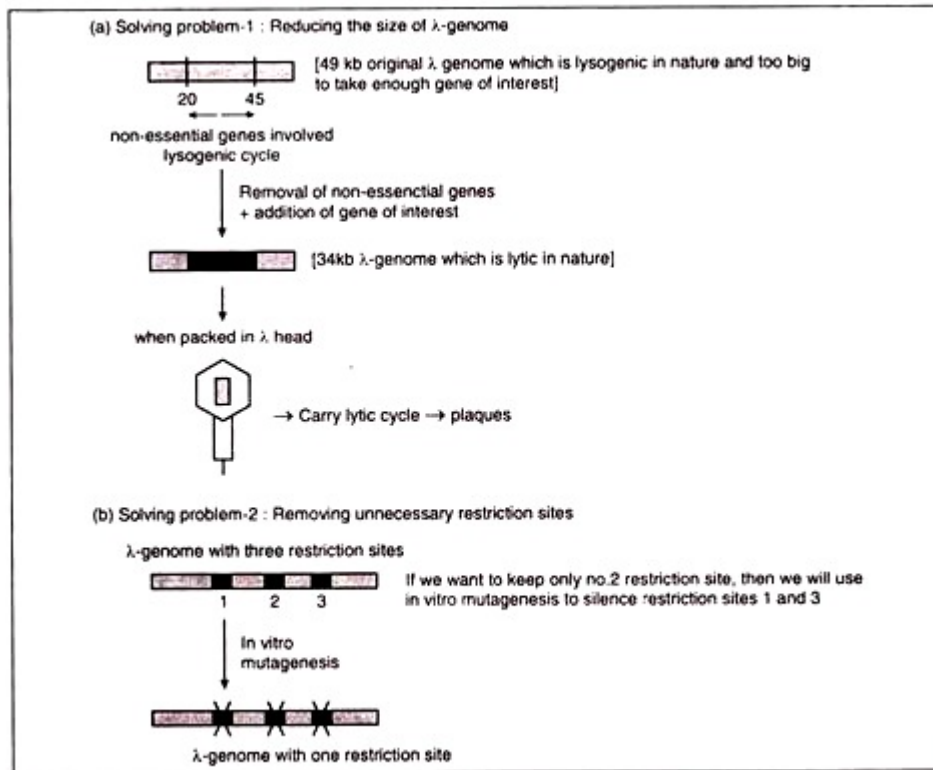


Fig. 4.31: Solutions of two problems associated with using lambda genome as cloning vector

Solving problem (2):

We can remove unnecessary restriction sites by carrying out in vitro mutagenesis. For example, an ECoRI site, GAATTC, could be changed to GGATTC, which is not recognized by the enzyme.

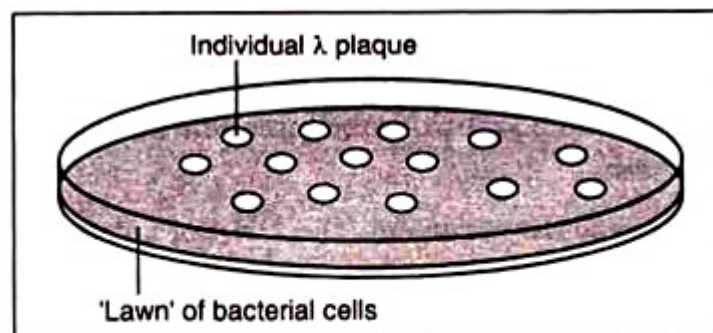


Fig. 4.32: The plaques formation due to the lytic cycle carried out by lambda phage

Types of Lambda Vectors:

There are two types of lambda cloning vectors.

(a) Lambda Insertion Vectors:

In this case a large segment of the non-essential region has been deleted, and the two arms ligated together. An insertion vector possesses at least one unique restriction site into which new DNA can be inserted. The size of the DNA fragment that an individual vector can carry depends on the extent to which the non-essential region has been deleted, e.g.; lambda-gt10, lambda-ZAP11.

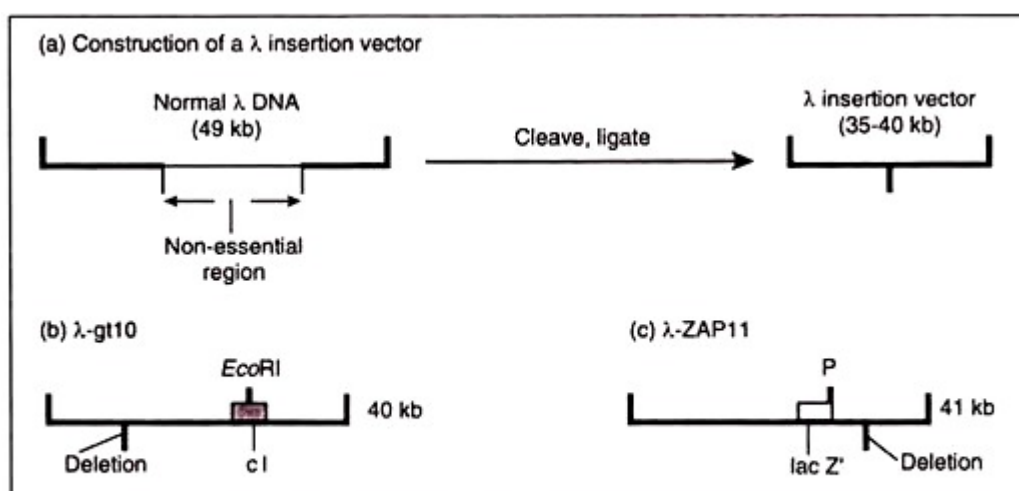


Fig. 4.33: Lambda insertion vectors. P = polylinker in the lac Z' gene of lambda ZAP II, containing unique restriction sites for SacI, NotI, XbaI, etc.

(b) Lambda Replacement Vectors:

These vectors have two recognition sites for the restriction endonucleases. These sites flank a segment of DNA that is replaced by the DNA to be cloned [Fig.4.34(a)]. Often the replaceable fragment (or stuffer fragment) carries additional restriction sites that can be used to cut it up into small pieces so that its own reinsertion during a cloning experiment is very unlikely.

Replacement vectors are generally designed to carry large pieces of DNA than insertion vectors can handle e.g., lambda- EMBL, lambda-GEM11, etc.

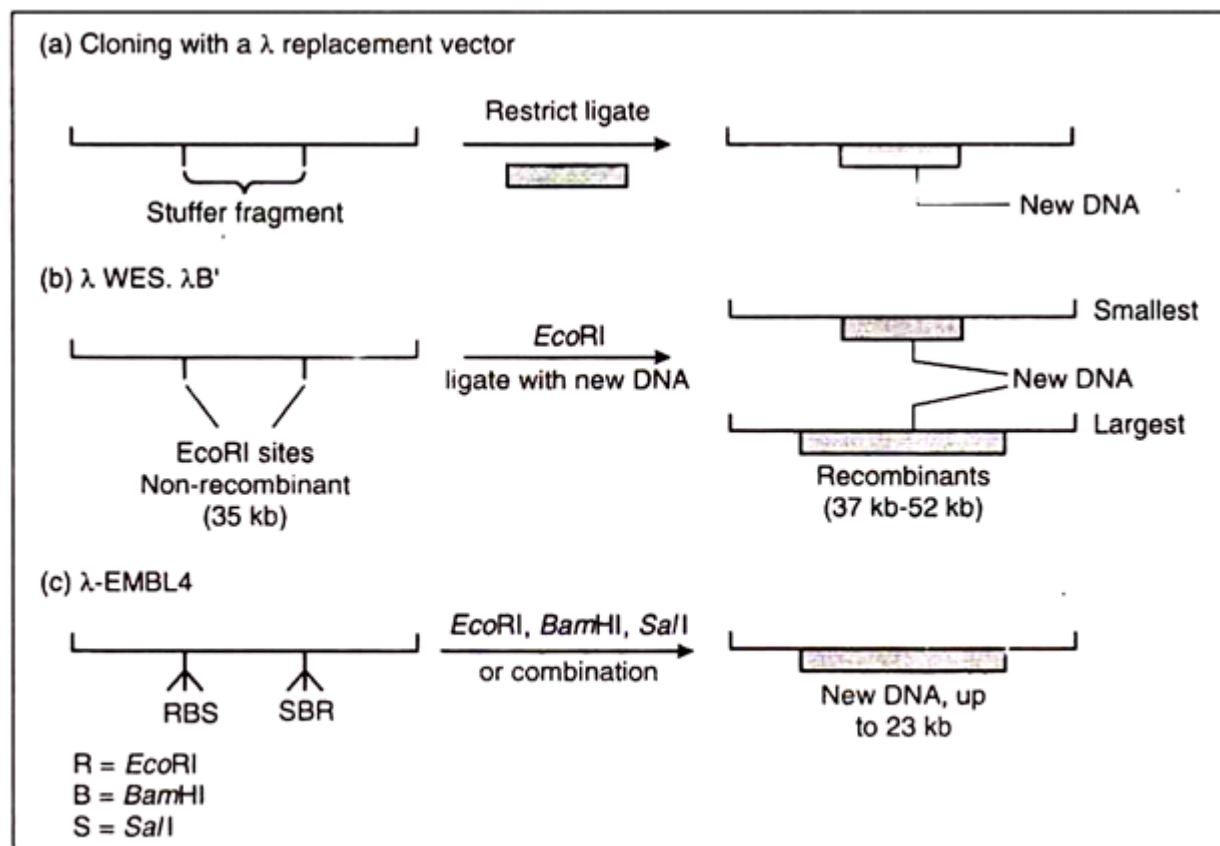


Fig. 4.34: Lambda replacement vectors

Cloning experiments with lambda insertion or replacement vectors:

A cloning experiment with a lambda vector can be carried out by following the similar method that we followed for a plasmid vector—the lambda DNA molecules are digested with suitable restriction endonuclease enzyme, the gene of interest is added, the mixture is ligated and the resulting recombinant DNA is introduced into *E. coli* host cell (by a process called transfection) [Fig. 4.35(a)].

This type of experiment requires that the vector be in its circular form, with the cos sites hydrogen bonded to each other.

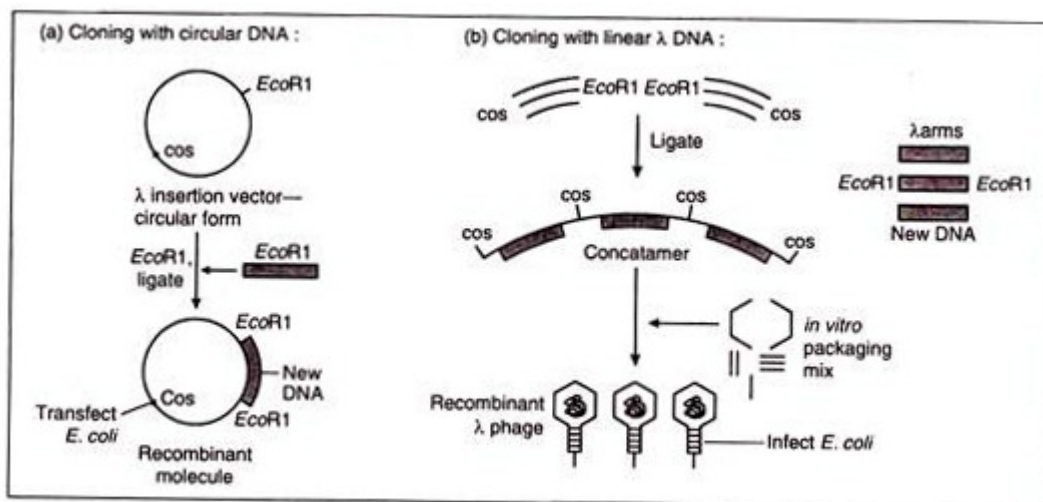


Fig. 4.35: Different strategies for cloning with lambda vector

The transfection process which requires a circular lambda DNA molecule is not particularly efficient. To obtain a greater number of recombinants we can introduce some refinements in the lambda genome. In this regard we can prefer a 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 DNA can be constructed by mixing together our gene of interest with the vector arms [Fig. 4.35(b)]. Ligation results in several molecular arrangements, including catenae's comprising left arm-DNA-right arm repeated many times. Recombinant phage thus produced in the test tube can be used to infect an E.coli culture.

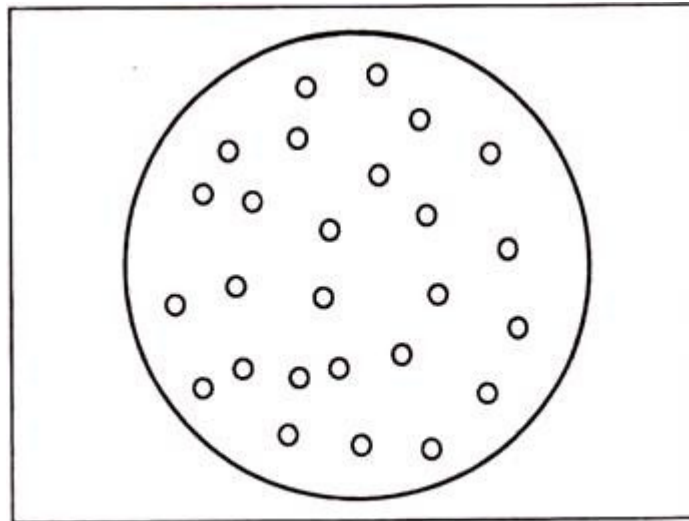


Fig. 4.36: Bacteriophage plaques

Visualization of Phage Infection after the Process of Transfection:

The entry of recombinant DNA in the host cell is followed by the lytic cycle which eventually results in the lysis of the host cell. The lysed host cell can be located on the agar medium as plaques on a lawn of bacteria. Each plaque is a zone of clearing produced as the phages lyse the cell and move on to infect the neighbouring bacteria.

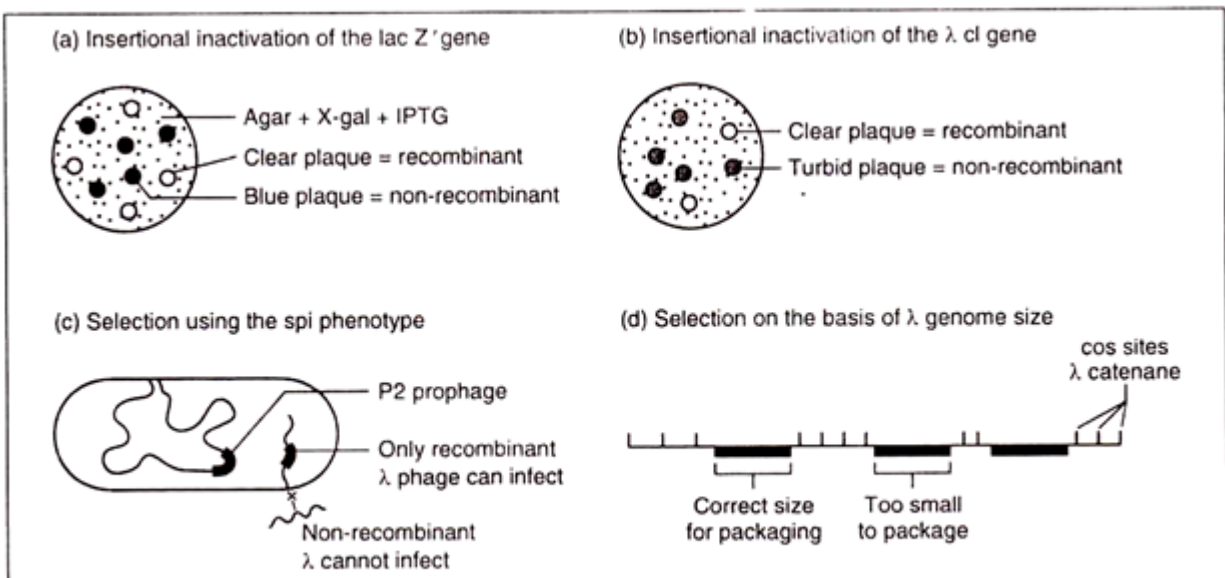


Fig. 4.37: Strategies for the selection of recombinant plaques using lambda vector

Screening of transformed host cells using bacteriophage lambda vectors:

A variety of ways could be employed to distinguish between recombinant plaques from non-recombinant ones.

The methods are as follows:

(a) Insertional Inactivation of Lac Z' Gene Carried by the Lambda Phage Vector:

Insertion of our gene of interest into the lac Z' gene inactivates beta-galactosidase synthesis. Recombinants are distinguished by plating cells on X-gal agar where the recombinant plaques are clear whereas non-recombinant plaques are blue in colour.

(b) Insertional Inactivation of Lambda Cl Gene:

Several lambda cloning vectors have restriction site in the cl gene. Insertional inactivation of cl gene cause a visible change in the plaque morphology. Normal plaques appear turbid (hazy) whereas recombinant plaques with disrupted cl gene are clear.

(c) Selection using Spi Phenotype:

P2 phage is a relative of lambda phage, lambda phages cannot infect E. coli cells that already has an integrated P2 phage in its genome. Due to this, lambda phage is said to be Spi⁺ (sensitive to P2 pro-phage infection). Some lambda cloning vectors are designed so that insertion of new DNA causes a change from Spi⁺ to Spi⁻, enabling the recombinant to infect cells that carry P2 prophages.

Such cells are used as host for cloning experiments with these vectors. In this case recombinants are Spi⁻, so they are able to form plaques.

(d) Selection on the Basis of Lambda Genome Size:

We know this from the beginning that any gene of interest which is less than 37kb or more than 52kb cannot be packed in the head of lambda phage. Many lambda vectors have been constructed by deleting large segments of the lambda DNA molecule and so are less than 37kb in length.

These can only be packaged into mature phage particles after our gene of interest has been inserted. This brings the total genome size up to 37kb or more. Hence, with these vectors only recombinant phages are able to replicate.

Uses of Bacteriophage Lambda Vectors:

The main use of all lambda based vectors is to clone DNA fragments that are too long to be handled by plasmid or M13 vectors. A replacement vector such as lambda-EMBL4 can carry up to 20kb of our gene of interest. This compares with a maximum insert size of about 8kb for almost all plasmids and less than 3kb for M13 vectors.

Advantages of Bacteriophage Lambda Vectors

Following are the advantages of lambda vectors:

1. Storage of phage particles is comparatively much easier than that of plasmid based vectors.
2. The shelf-life of phage particles is infinite.
3. Transfection of E. coli is much easier with phage particles.

Disadvantages of Bacteriophage Lambda Vectors:

If you have isolated a clone, it is frequently quite difficult to isolate large quantities of DNA. In practice, many problems are encountered that do not occur with plasmids. There is still no truly rapid, reliable protocol for the production of very clean lambda-DNA.

The most successful method is to use anion exchange columns. The most frequent problem is that the preparation contains dirt that makes further processing, such as a restriction digestion, difficult or impossible.

Even in the replacement vectors, almost two thirds of the DNA is made up of vector sequences. If possible, you should clone the sections that are of interest using plasmids. LambdaZAP banks can save work, because the plasmid portions are cut out *in vivo*, along with inserted DNA. That process is highly efficient, requires only a relatively few work steps, and lasts only 1 to 2 days.

λ GTII	λ insertion vector Insert capacity 7.2 kbp <i>lac Z</i> gene	cDNA library construction Expression of inserts	Various
λ EMBL3/4	λ replacement vectors Insert capacity 9-23 kbp	Genomic library construction	Various
λ ZAP Express®	λ -based insertion vector Capacity of 12 kbp <i>In vivo</i> excision of inserts Expression of inserts	cDNA library construction Also genomic/PCR cloning	Stratagene
λ FIX®II	λ -based replacement vector; capacity 9-23 kbp Spi ^r /P2 selection system to reduce non-recombinant background	Genomic library construction	Stratagene
pBluescript®II	Phagemid vector Produces single-stranded DNA	<i>In vitro</i> transcription DNA sequencing	Stratagene
SuperCos I	Cosmid vector with Ap ^r and Neo ^r markers, plus T3 and T7 promoters Capacity 30-42 kbp	Generation of cosmid-based genomic DNA libraries T3/7 promoters allow end-specific transcripts to be generated for chromosome walking techniques	Stratagene

III. Cosmid Vectors:

It is the most sophisticated type of lambda based vector. Cosmids are the hybrids between the phage DNA molecule and bacterial plasmid. Their design centres on the fact that the enzymes that package the lambda DNA molecule into the phage protein coat need only the *cos* sites in order to function.

Construction of Cosmid Vectors:

A cosmid is basically a plasmid that carries a *cos* site. It also needs a selectable marker, such as ampicillin resistant gene, and a plasmid origin of replication.

This is important to note that as cosmid lacks all the lambda genes, so it does not produce plaques. Instead colonies are formed on the selective media just as with plasmid vectors.

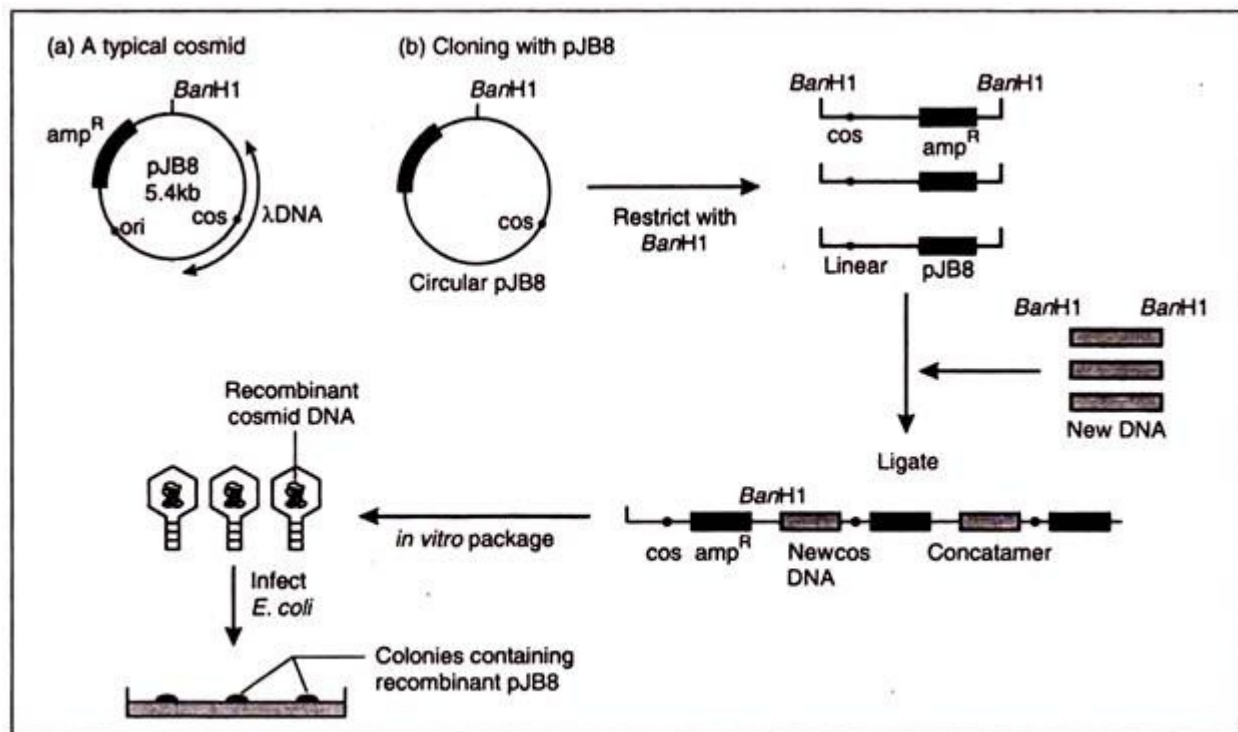


Fig. 4.38: A typical Cosmid vector and the way it is used to clone long fragments of DNA

Cloning Experiment with Cosmid Vectors:

This is carried out as follows. The cosmid is opened and its unique restriction site and our gene of interest is inserted. These fragments are usually produced by partial digestion with a restriction endonuclease, as total digestion almost invariably results in fragments that are too small to be cloned with a cosmid.

Ligation is carried out so that catenanes are formed. These lambda phages are then used to infect an E. coli culture. All colonies are recombinant colonies as non-recombinant lambda phages cannot be packaged into the head of the lambda bacteriophage.

Uses of Cosmid Vectors:

Cosmids are used for construction of genomic libraries of eukaryotes since these can be used for cloning large fragments of DNA.

Advantages of Cosmids:

Followings are advantages of cosmid vectors:

1. These can be used to clone gene of interest up to 40 kb.
2. As the lambda phage will insert the recombinant DNA into the host cell, an extra step of inserting the recombinant DNA into the host cell is not performed.
3. Easy screening method is found.

The Phagemid Vectors:

Although M13 vectors are very useful for the production of single-stranded versions of cloned genes they do have one disadvantage. There is a limit to the size of DNA fragment that can be cloned with an M13 vector, with 1500bp generally being looked on as the maximum capacity.

To get around this problem a number of novel vectors have been constructed which are the hybrids of plasmids and M13 vectors. We call them phagemids ('phage' from M13 bacteriophage and 'mid' from plasmid).

Construction of Phagemid Vector:

A typical phagemid has following parts:

1. Phage M13 origin of replication.
2. A portion of lac Z' gene driven by lac promoter.
3. A multiple cloning site (MCS) with lac Z' gene.

4. Phage T7 and T3 promoter sequences flanking the MCS sequences.
5. ColE1 origin of replication.
6. amp^R resistant gene.

Plasmids that carry the M13 replication origin in addition to a conventional origin of dsDNA synthesis can be replicated either as dsDNA from the latter or as single-stranded DNA from the M13 origin. Replication from the M13 origin requires the appropriate proteins (such as gene II protein) to be provided from a helper phage also replicating within the cell.

Replication generates single-stranded DNA which can then be packaged into phage coats. Examples of phagemids are the vectors pUC118, 119 and 120.

They are replicated as plasmids until the cell containing them is co-infected with a helper phage, such as M13K07, which provides the proteins for single-stranded DNA synthesis and packaging. M13K07 is an M13 phage that has been modified, most importantly by the incorporation of a plasmid replication origin.

Replication from this origin allows the helper phage to be present in a high copy number per cell and, therefore, to provide the larger quantities of the proteins that are required to replicate and package the phagemid molecule.

M13K07 also contains a kanamycin resistance gene to allow for selection for the presence of the helper phage. (Of course, it is possible that the M13K07 helper phage may be packaged too, but in practice the packaged phagemid molecules are found to be in a 100-fold excess over the helper phage.)

Another example of these vectors is the pBluescript series, such as pBluescriptIIKSP, shown in Fig. 4.39. This series of plasmids contains, in addition to features already described, promoters from the E. coli bacteriophages T3 or T7, which are useful for expressing cloned sequences.

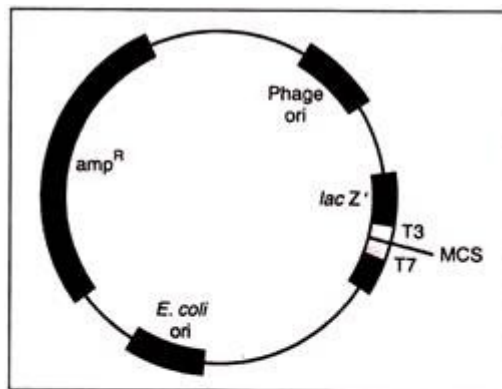


Fig. 4.39: pBluescriptIIKSp (3.0 kb). The phagemid contains an ampicillin resistance selectable marker (amp^R) and origins (ori) for double-stranded replication (*E. coli*) and for single-stranded DNA synthesis (Phage), the latter for use when cells containing the vector are co-infected with a suitable helper phage. There are a multiple cloning site (MCS) in the $lacZ'$ minigene (allowing blue-white selection for the presence of an insert) and phage T3 and T7 promoters for transcription of inserted DNA sequences

Uses of Phagemid Vectors:

This vector is a multipurpose vector as it can serve as following:

1. A cloning vector.
2. An expression vector
3. A sequencing vector.

Advantages of Phagemid Vectors:

The main advantage of the phagemid system is that it can be used to provide single-or double-stranded material without any re-cloning.

Phasmid Vectors:

Phasmids are truly plasmids with phage genes. These are linear duplex DNAs whose ends are lambda segments that contain all the genes required for a lytic infection and whose middle-portion is linearized. Both the lambda and the plasmid replication functions are intact.

Normally, plasmid vectors carry a lambda attachment site. Once inside E. coli cell, the phasmid can replicate like a phage and form plaques in the normal way. However, if the vector contains the gene that encodes lambda repressor, then the plasmid replicates as a plasmid rather than as a phage.

Depending upon the functioning or non-functioning of cI - Protein (coded by repressor), the phasmid can replicate as plasmid (cI -Protein inactive) or phage when cI -protein is active. The activity of cI -protein can be inactive by growing the E.coli culture at 40°C .

Plasmids may be used in variety of ways. For example, DNA may be cloned in the plasmid vector in a conventional way and then the recombinant plasmid can be lifted onto the phage. Plasmids are easy to store, they have an effectively infinite shelf life and screening phages by molecular hybridization gives cleaner results than screening bacterial colonies.

Eukaryotic Vectors:

Most cloning experiments are carried out with E. coli as the host, and the widest variety of cloning vectors are available for this organism. E. coli is particularly popular when the aim of the cloning experiment is to study the basic features of molecular biology such as gene structure and function. However, under some circumstances it may be desirable to use a different host for a gene cloning experiment.

But when the aim of the RDT experiment is not just to study a gene but to use cloning to control or improve synthesis of an important metabolic product (e.g., a hormone such as insulin), or to change the properties of the organism (e.g., to introduce herbicide resistance into a crop plant), then we take a host cell which is more advanced and capable of meeting an advanced level of metabolism.

Due to this, many times we consider eukaryotic vectors for our cloning experiments. Yeast, animal and plant vectors are all considered as eukaryotic vectors.

Table 4.6: Different types of cloning vectors and the size of the inserted DNA that can be accommodated

Types of Vector	Insert DNA size (in kb)
Plasmid	0.5 to 8
Bacteriophage Lambda	9 to 25
Cosmid	30 to 45
YAC	250 to 1000
BAC	50 to 300

Yeast Vectors:

The yeast (*Saccharomyces cerevisiae*) is one of the most important organisms in biotechnology. Its role is also very important in brewing and bread making. Yeast has been used as a host organism for the production of important pharmaceuticals from cloned genes. Development of cloning vectors for yeast has been stimulated greatly by the discovery of plasmid that is present in most strains of *S. cerevisiae*.

General Construction:

Various yeast vectors have been designed, once the ability and utility of yeast is confirmed. All of them have three features in common.

1. All of them contain unique target sites for a number of restriction endonucleases.
2. All of them can replicate in *E. coli* often at high copy number.
3. All of them employ markers that can be used to select recombinant yeasts, e.g., *Hi53*, *leo2*, *trp1* and *ura3*.

Types of Yeast Vectors:

All the yeast vectors can be divided into three types:

1. Yeast cloning vectors (or Yeast plasmid vectors)
2. Yeast expression vectors
3. Yeast artificial chromosomes (YAC)

Yeast Cloning Vectors (or Yeast Plasmid Vectors):

These vectors are used to clone (make several duplicate copies) our gene of interest in the yeast host cell. All the cloning vectors have been engineered from 2 μ x plasmid which is the naturally occurring plasmid in the yeast cell.

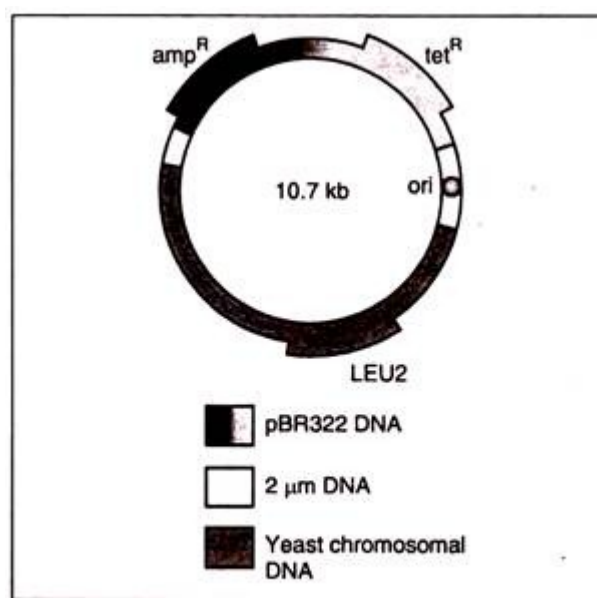


Fig. 4.40: Structure of YEps

They are of following types:

1. Yeast Episomal Plasmids (YEps):

It is 6,318 bp long and has a copy number of 70-200. Most of the YEps are shuttle vectors (can be used as vectors both in prokaryotic hosts and eukaryotic hosts) and thus have been engineered accordingly. An example of YEps is Yep13.

It have following parts:

- (a) Origin of replication derived from 2m plasmid.
- (b) amp^{R} and tet^{R} region from pBR322. This selectable marker region is helpful for the screening of recombinants host cells when the vector is used in a prokaryotic cell.
- (c) LEU2 region which is derived from yeast chromosome and could be used as a selectable when the vector is used in an eukaryotic cell. LEU2, which codes for beta-iso-propyl-malate dehydrogenase, one of the enzymes involved in the conversion of pyruvic acid to leucine.

This is very important to note that when we are taking yeast cells for YEps then we have to take only leu2 yeasts which must be auxotrophic mutants having non-functional LEU2 gene.

Screening Process:

The leu2^- yeast host cells are unable to synthesize amino acid leucine and can survive, only if this amino acid is supplied as a nutrient in the growth medium [Fig. 4.41(a)]. Selection is possible because transformants host cells contain a copy of YEp (having LEU2 gene) and are quite able to grow in the absence of amino acid.

In a cloning experiment, cells are plated out onto minimal medium, which contains no added amino acids. Only transformed cells are able to survive and form colonies [Fig. 4.41(b)].

YEp may get inserted into the yeast chromosome by a process of homologous recombination between the plasmid LEU2 gene and the yeast mutant LEU2 gene. The plasmid may remain integrated, or a later recombination event may result in it being excised again.

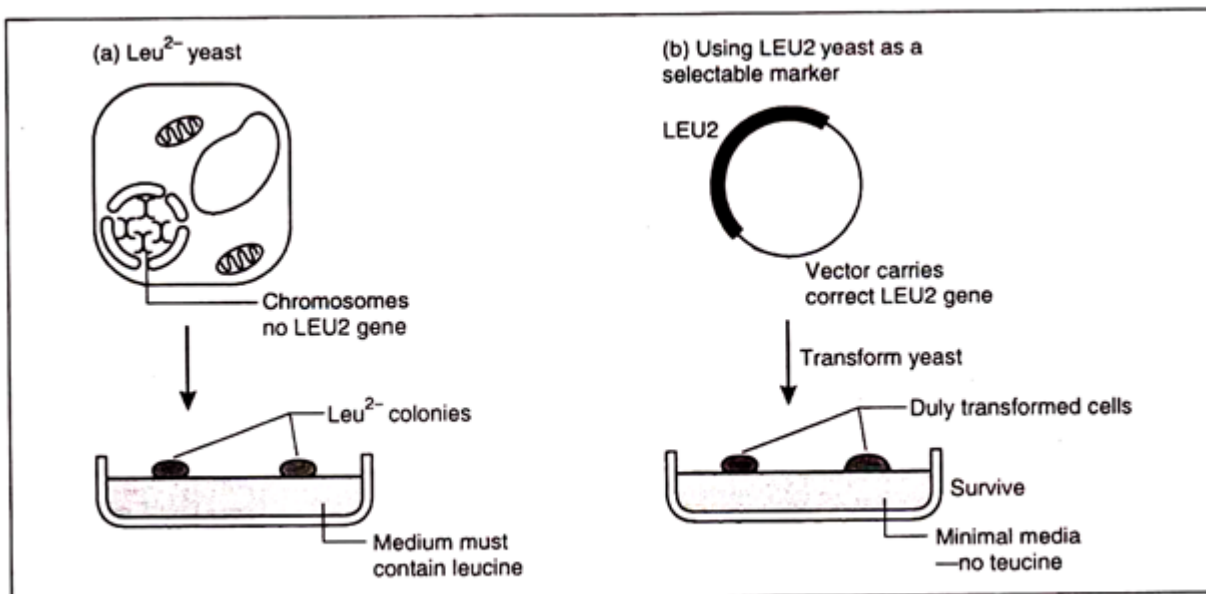


Fig. 4.41: Using the $LEU2$ gene as a selectable marker in a yeast cloning experiment

2. Yeast Integrative Plasmids (YIps):

These are basically bacterial plasmids carrying a yeast gene. An example is YIp5, which is pBR322 with an inserted $URA3$ gene.

3. Yeast Replicative Plasmids (YRps):

These are able to multiply as independent plasmids because they carry a chromosomal DNA sequence that includes an origin of replication. An example is YRp7.

Yeast Expression Vectors:

These vectors are used when our aim is to express our gene of interest in the yeast cell. Yeast expression vectors will employ promoter and terminator sequences in addition to the gene of interest. Apart from these we have genetic tags like the gene for green fluorescent protein (GFP) for tracking the location of the protein after its biosynthesis.

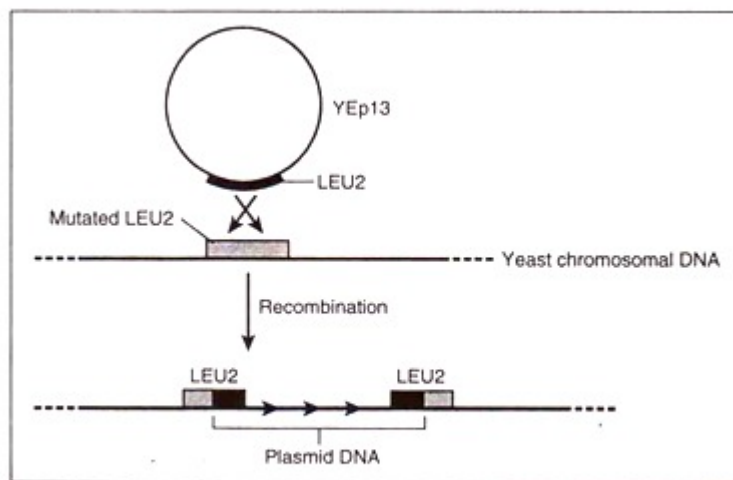


Fig. 4.42: Recombination between plasmid and chromosomal *LEU2* genes can integrate YEpl3 into yeast chromosomal DNA

Following are few examples:

(a) p427-TEF:

High copy yeast expression vector carrying the aminoglycoside phosphotransferase gene for selection in yeast using G418. Inserts are expressed from the strong TEF promoter.

(b) p417-CYC:

Low copy yeast expression vector carrying the aminoglycoside phosphotransferase gene for selection in yeast using G418. Inserts are expressed from the weak CYC1 promoter.

(c) PTEF-MF:

Yeast expression vector for secreted proteins. A strong TEF1 promoter drives constitutive expression of a cDNA fused to the pre-pro leader sequence of mating factor alpha to ensure secretion of the protein product into the medium.

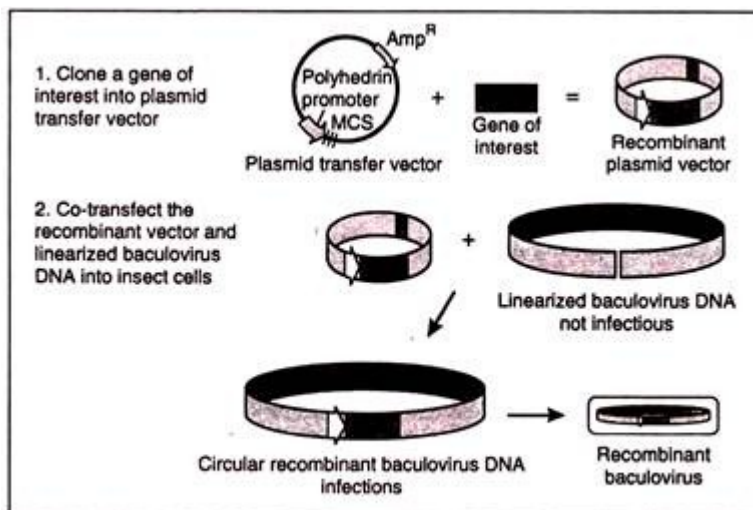


Fig. 4.43: Generation of recombinant Baculovirus

Yeast Artificial Chromosomes:

Yeast artificial chromosomes (YACs) are synthetic double-stranded linear constructs containing the elements necessary for replication as independent chromosomes in yeast. See artificial chromosomes for more details.

Animal Vectors:

The followings are some examples of animal vectors:

1. Baculovirus Vector:

Baculovirus infects insects. This virus is rod shaped with a large double-stranded genome. During normal infections, baculovirus produces nuclear inclusion bodies which consist of virus particles embedded in a protein matrix.

This protein matrix is called polyhedrin and it accounts for 70% of total protein encoded by the virus. Genetic manipulation of the viral DNA is not possible as it has a very large DNA with many restriction sites for a single enzyme.

Hence, the gene of interest is cloned into the small recombination transfer vector and co-transfected into insect cell lines along with the wild type of virus

in the cell. Homologous recombination takes place between the polyhedrin gene and our gene of interest.

Thus, our gene of interest will be transferred from the vector plasmid into the wild type of virus and polyhedrin gene will be transferred from the virus on the plasmid.

This is something like displacement reaction. This displacement of gene will not affect the replication of virus, as polyhedrin gene is not required for replication. The recombination virus replicates in the cells and generates characteristic plaques (without inclusion bodies).

Normally the virus is cultured in the insect cell line of *Spodoptera frugiperda*. The gene of interest is expressed during the infection and very high yields of protein can be achieved by the time the cell lyses.

2. Bovine Papilloma Virus Vector:

Bovine papilloma virus (BPV) causes warts (uncontrolled epithelial proliferation) in cattle.

BPV normally infects terminally differentiated squamous epithelial cells.

BPV has a capsid protein surrounding a circular double-stranded DNA of size 79 kb. 69% of this genome is important for viral function, whereas 31 % of the genome can be replaced by any foreign DNA sequence like our gene of interest.

The recombinant BPV is constructed by ligating our gene of interest and BPV vector (69%) onto the pBR 322 plasmid, thus generating the shuttle vector containing plasmid ori site and virus replication sequences. These shuttle vectors are multiplied in *E. coli* cells first and then they are transformed into mouse cell line.

The major advantage of BPV is the generation of permanent cell line. As the infected cells are not killed, a stable plasmid number is found even when the insert is of large size. The selection of transform-ants is very easy as they form a pile of cells on the transferred monolayer of cells called “**Focus**”.

The transformed cells are then selected by the presence of marker gene which is mostly the neomycin phospho transferase gene coding for resistance against G418. Example of this type of vector is p3.7LDL.

3. SV40 Virus based Vectors:

SV 40 is a spherical virus with double-stranded circular DNA of size 5.2 kb. The viral protein contains three viral coded proteins. VP1 is the major protein present in the capsid with a size of 47000 kDa. Two more proteins VP2 and VP3 are also present.

The DNA of virus is associated with the four histones (H4, H2A, H2B and H3) proteins. The viral DNA can be segmented into five precise segments coding for five different proteins small T, large T, VP1, VP2 and VP3. VP1 coding region overlaps VP2 and VP3 in a different translation reading frame. SV 40 virus infects monkey kidney cell lines.

The virus travels to the nucleus and gets uncoated. Then both the T-genes located near the origin are translated in the clockwise direction. The large T protein is important for virus DNA replication and starts after the translation of large T -protein.

Replication starts at the origin and is bi-directional. It terminates when two replication forks meet. About 10⁵ molecules of duplex DNA are synthesized per cell. Along with DNA replication, VP1, VP2 and VP3 proteins are synthesized.

Then packing of DNA occurs to form new virions, which are released by the lysis of cell. The entire process can also be initiated by transfection with naked

SV 40 DNA. SV 40 vectors are constructed similar to phage vectors. Portions of the viral genome are removed and replaced by other DNA segments. There are three types of SV 40 vehicles each of which have a distinct advantage or disadvantage among themselves.

They are as follows:

(a) SV40 Passive Transforming Vectors:

These vectors neither replicate nor produce virions, but simply integrate the DNA segments into the cellular DNA. These transformed cells replicate the new DNA as an integral part of their own genomes. These plasmids are also shuttle vectors and include selective markers like herpes virus, thymidine kinase or neo genes.

Apart from the selective markers, they include transcriptional regulator signals and polyadenylation sites.

(b) SV40 Trans-ducting Vectors:

These vectors are capable of replicating and packing into virion particles. Transducing vectors contain a segment of 300 bp which functions as the origin of replication and provides the transcriptional regulatory signals for the synthesis of mRNAs.

This type of vector takes an insert of size 3.9 to 4.5 kb. These plasmids do not have the genes that code for VP1, VP2 and VP3. As no DNA can be added to SV 40 DNA without removing any DNA from the genome, to add the insert, the genome DNA that is not required is removed.

The functions of the DNA that are lost by these deletions are supplied by using a helper virus or by inserting the SV 40 deleted genes into the host DNA. Normally the recombinant SV 40 vectors (usually consist of DNA of interest

and replication sequence and gene for coding VP1, VP2 and VP3) are transformed into the cos cell line.

Cos cell line is a kidney cell line of the African green monkey kidney. It has the T-protein gene incorporated in the genome. So when the vector is transfected into these cells, virion particles are yielded with the help of helper virus.

(c) SV40 Plasmid Vectors:

These vectors multiply in the monkey cell line but are not packed as the virions. These vectors usually contain origin of replication sequences and larger T-protein gene but do not contain VP1, VP2 and VP3 genes. They are shuttle vectors, and have the ability to multiply both in E. coli and monkey cell line.

Plant Vectors:

Cloning vectors for higher plants were developed in 1980s and their use has led to the genetically modified (GM) crops that are in the headline today.

We will examine the details of plant vectors and the genetic modification of crops. Here we look at the cloning vectors and how they are used.

Three types of cloning system have been used with varying degrees of success with higher plants:

1. Vectors based on naturally occurring plasmids of Agrobacterium (e.g., Ti plasmids from *A. tumefaciens* and Ri plasmid from *A. rhizogens*).
2. Direct gene transfer using various types of plasmid DNA. (e.g., using of supercoiled plasmids).
3. Vectors based on plant viruses (e.g., Caulimovirus vectors and Gemini virus vectors).

Shuttle vectors:

Shuttle vectors are those which can multiply into two different unrelated species. Shuttle reactors are designed to replicate in the cells of two species, as they contain two origins of replication, one appropriate for each species as well as genes that are required for replication and not supplied by the host cell, i.e., it is self-sufficient with the process of its replication.

The shuttle vectors are of following types:

1. Eukaryotic – Prokaryotic Shuttle Vectors:

Vectors that can propagate in eukaryotes and prokaryotes. e.g., YEp vectors can be propagated in yeast (fungi) as well as in E. coli (bacteria).

2. Prokaryotic – Prokaryotic Shuttle Vectors:

Vectors that can be propagated in two unrelated prokaryotic host cells, e.g., RSF1010 vectors can be propagated both in bacteria as well in spirochetes.

The common features of such shuttle vectors or eukaryotic vectors are the following:

- (a) They are capable of replicating into two or more types of hosts including prokaryotic and eukaryotic cells.
- (b) They replicate autonomously, or integrate into host genome and replicate when the host cell multiplies.
- (c) These vectors are commonly used for transporting genes from one organism to another.

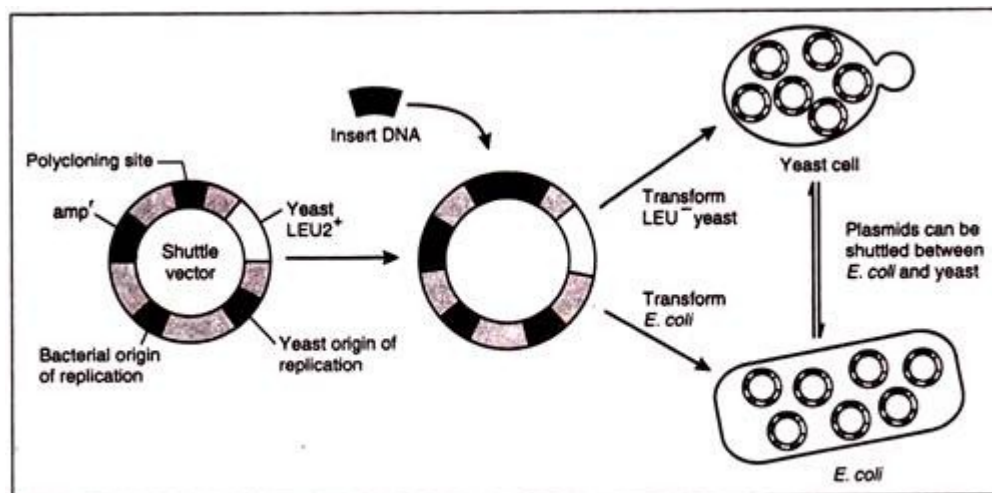


Fig. 4.44: An yeast-bacteria shuttle vector

Disadvantage:

The presence of two replication origins sometimes poses special problems, one portion of replication origin of one species is totally unrelated to another and interferes with the replication of other host. Hence, in a shuttle vector various types of replication origins are to be inserted and checked before experimenting.

Artificial Chromosomes:

Artificial chromosomes are synthetically designed DNA molecules of known structure, which are assembled in vitro (in the laboratory) from specific DNA sequences that acts like a natural chromosome. Artificial chromosomes are circular or linear vectors that are stably maintained in, usually, 1-2 copies per cell.

They are huge in size in comparison to other vectors but can clone very large segments of chromosomes (even an entire chromosome). Before seeing different types of artificial chromosomes the three key components of an eukaryotic chromosomes can be seen which are necessary for its stable maintenance inside a cell.

1. The centromere, which is required for the chromosome to be distributed correctly to daughter cells during cell division.
2. Two telomeres, the structures at the ends of a chromosome, which are needed for the ends to be replicated correctly in order and which also prevent the chromosome from being nibbled away by exonucleases.
3. The origin of replication, which are the positions along the chromosome at which DNA replication initiates, similar to the origin of replication of a plasmid.

Once we have defined the chromosomal structure of an eukaryotic organism (like humans and yeast), then we can isolate the key components of their chromosomes and join them together to form an artificial chromosome. Then into this artificial chromosome we can insert our gene of interest which can be subsequently cloned in its respective cell.

Following are the types of artificial chromosomes:

A. Yeast Artificial Chromosomes (YAC):

A YAC can be considered as a functional artificial chromosome, since it includes three specific DNA sequences that enable it to propagate from one yeast cell to its offspring:

1. The Telomere (TEL):

The telomere which is located at each chromosome end, protects the linear DNA from degradation by Nucleases.

2. The Centromere(CEN):

The centromere which is the attachment site for mitotic spindle fibres, “pulls” one copy of each duplicated chromosome into each new daughter cell.

3. Origin of Replication(OR):

Replication origin sequences which are specific DNA sequences that allow the DNA replication machinery to assemble on the DNA and move at the replication forks.

4. A and B:

Selectable markers that allow easy isolation of yeast cells that have taken up artificial chromosome.

5. Recognition Site:

Recognition Site for two restriction enzymes EcoRI and BamHI.

Cloning experiment using a YAC vector:

1. Large DNA fragments are obtained by carrying out restriction digestion using EcoRI.
2. The YAC is digested by two restriction enzymes EcoRI and Bam HI.
3. Those two elements recombine at the EcoRI sites and are covalently linked by DNA ligase.
4. A recombinant YAC vector, a yeast artificial chromosome with genomic DNA inserted, is produced. This vector can be used to infect yeast cells and generate an unlimited number of copies.

Uses of YAC Vectors:

1. YAC can be used to study various aspects of chromosome structure and behaviour; for instance, to examine the segregation of chromosomes during meiosis.

2. YAC cloning system can take DNA insert greater than 100kb. Due to this they can be used to study the functions and modes of expression of genes that had previously been intractable to analysis by recombinant DNA techniques.

3. YACs can be propagated in mammalian cells, enabling the functional analysis to be carried out in the organism in which the gene normally resides. Thus by using them we can learn about the true form of gene expression in vivo conditions.

4. Yeast artificial chromosomes are very helpful in the production of gene libraries. E. coli vectors can take DNA insert maximum up to 300kb. Due to this some 30000 clones are needed for a human gene library if we use them as cloning vector.

However, YAC vectors are routinely used to clone 600 kb fragments, and special types are able to handle DNA up to 1400 kb in length, the latter bringing the size of a human gene library down to just 6500 clones.

Sometimes YAC is seen with problem of lacking insert stability, the cloned DNA sometimes becoming rearranged by intra-molecular recombination. Nevertheless, YACs have been of immense value in providing long pieces of cloned DNA for use in large scale DNA sequencing projects. Example of YAC is pYAC3.

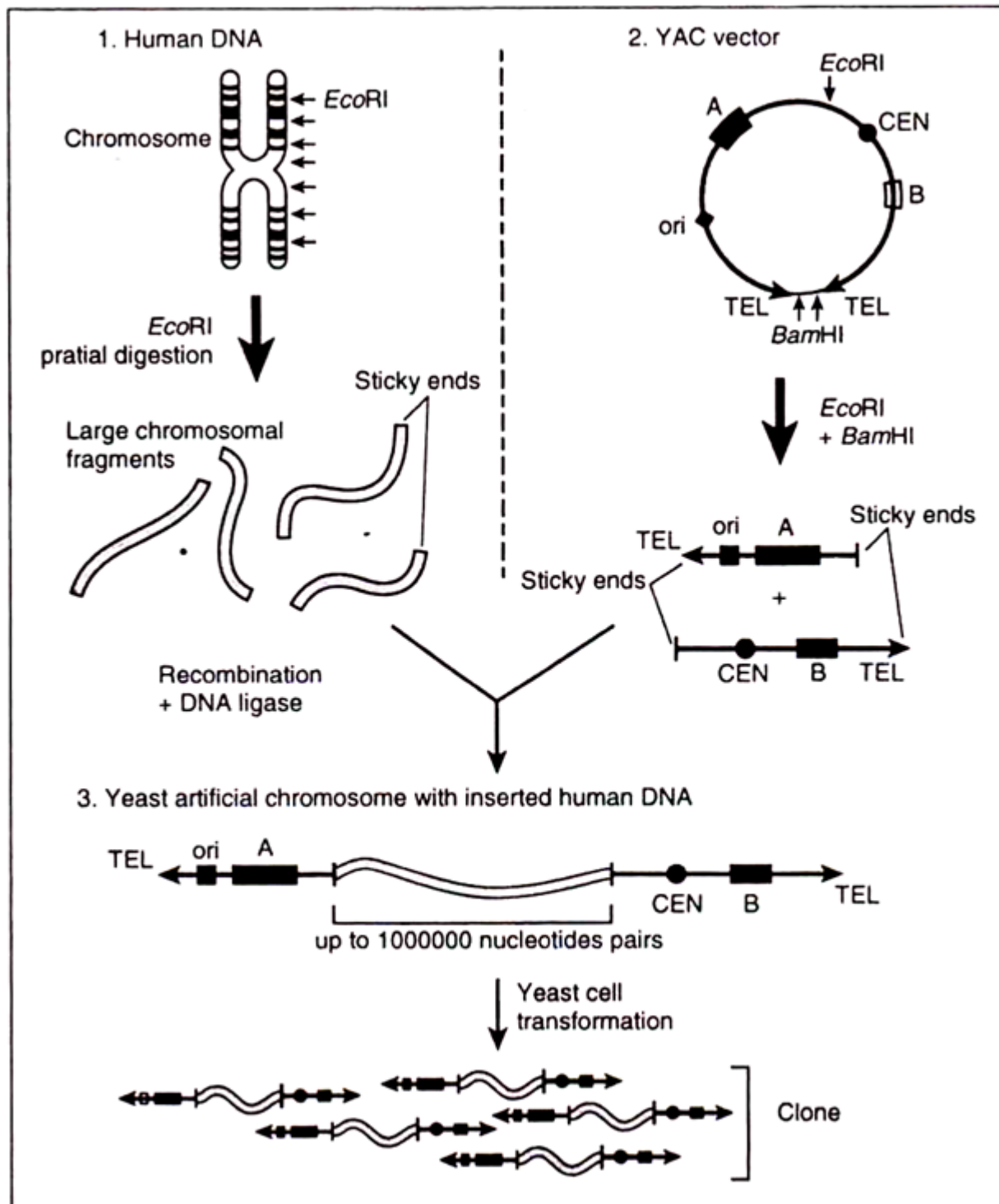


Fig. 4.45: The steps involved in cloning a large segment of human DNA in YAC

2. Bacterial Artificial Chromosomes (BAC):

Bacterial Artificial Chromosomes (BACs) are cloning vectors based on the extra-chromosomal plasmids of *E. coli*, called F factor or fertility factor. These

vectors enable the construction of artificial chromosomes, which can be cloned in *E.coli*.

This vector is useful for cloning DNA fragments up to 350 kb, but can be handled like regular bacterial plasmid vectors, and is very useful for sequencing large stretches of chromosomal DNA.

Like any other vector, BACs contain *ori* sequences derived from *E. coli* plasmid F factor, multiple cloning sites (MCS) having unique restriction sites, and suitable selectable markers. The genomes of several large DNA viruses and RNA viruses have been cloned as BACs. These constructs are referred to as “**infectious clones**”, as transfection of the BAC construct into host cells is sufficient to initiate viral infection.

The infectious property of these BACs has made the study of many viruses such as herpes viruses, poxviruses and coronaviruses more accessible. BACs are now being utilized to a greater extent in modelling genetic diseases, often alongside transgenic mice.

BACs have been used in this field as complex genes may have several regulatory sequences upstream of the encoding sequence, including various promoter sequences that will govern a gene's expression level. BACs have been used to some degree of success with mice when studying neurological diseases such as Alzheimer's disease or as in the case of aneuploidy associated with Down's syndrome.

Examples of BAC are pBACe3.6, pBeloBAC11 etc.

3. Human Artificial Chromosomes (HAC):

A human artificial chromosome (HAC) is a mini-chromosome that is constructed artificially in human cells. That is, instead of 46 chromosomes, the cell could have 47 with the 47th being very small, roughly 6-10 mega-bases in size, and able to carry new genes introduced by human researchers.

Using its own self-replicating and segregating systems, a HAC can behave as a stable chromosome that is independent of the chromosomes of host cells.

The essential elements for chromosome maintenance and transmission are the following three regions:

1. The “replication origin,” from which the duplication of DNA begins,
2. The “**centromere**,” which functions in proper chromosome segregation during cell division, and
3. The “telomere,” which protects the ends of linear chromosomes.

They are useful in expression studies as gene transfer vectors and are tools for elucidating human chromosome function. Grown in HT1080 cells, they are mitotically and cytogenetically stable for up to six months.

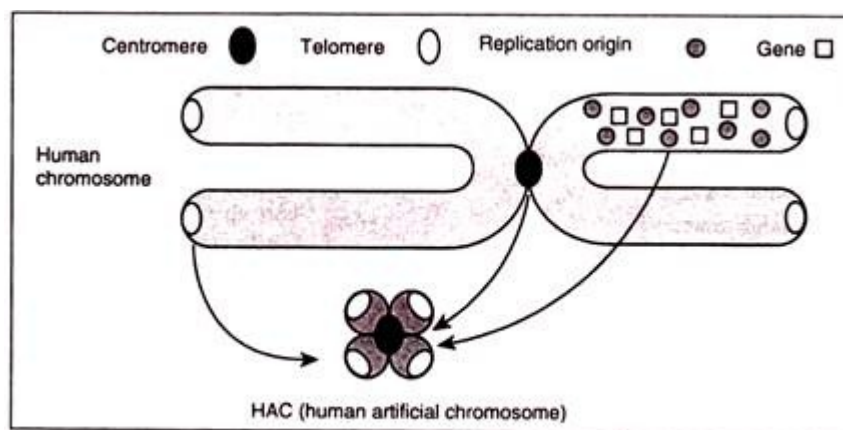


Fig. 4.46: HAC is generated by collecting necessary sequences from the human chromosome

4. Pl-Derived Artificial Chromosome (PAC):

These are DNA constructs which are derived from the DNA of PI bacteriophage. They can carry large amounts (about 100-300 kilo-bases) of other sequences for a variety of bio-engineering purposes. It is one type of vector used to clone DNA fragments (100- to 300-kb insert size; average, 150 kb) in *Escherichia coli* cells.

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