Unit 2, Molecular Cloning Tools and Strategies

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MICROBIOLOGY, SEM 4 CC10, Unit-2

Unit 2. Molecular cloning tools and strategies

2-1.1 Restriction enzyme:

A restriction enzyme is a nuclease enzyme that cleaves DNA sequence at a random or specific recognition sites known as restriction sites. In bacteria, restriction enzymes form a combined system (restriction + modification system) with modification enzymes that methylate the bacterial DNA. Methylation of bacterial DNA at the recognition sequence typically protects the own DNA of the bacteria from being cleaved by restriction enzyme.

There are two different kinds of restriction enzymes:

(1) **Exonucleases** catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5'to 3' direction or 3' to 5' direction. Example: exonuclease I, exonuclease II etc.

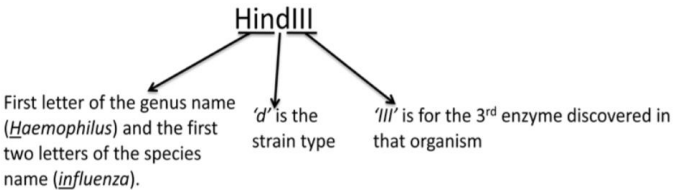
(2) **Endonucleases** can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. Example: EcoRI, Hind III, BamHI etc.

2-1.2History:

In 1970 the first restriction endonuclease enzyme HindII was isolated. For the subsequent discovery and characterization of numerous restriction endonucleases, in 1978 Daniel Nathans, Werner Arber, and Hamilton O. Smith awarded for Nobel Prize for Physiology or Medicine. Since then, restriction enzymes have been used as an essential tool in recombinant DNA technology.

2-1.3 Restriction Endonuclease Nomenclature:

Restriction endonucleases are named according to the organism in which they were discovered, using a system of letters and numbers. For example, HindIII (pronounced "hindee-three") was discovered in *Haemophilus influenza* (strain d). The Roman numerals are used to identify specific enzymes from bacteria that contain multiple restriction enzymes indicating the order in which restriction enzymes were discovered in a particular strain.



2-1.4 Classification of Restriction Endonucleases:

There are three major classes of restriction endonucleases based on the types of sequences recognized, the nature of the cut made in the DNA, and the enzyme structure:

• Type I restriction enzymes

• Type II restriction enzymes

• Type III restriction enzymes

2-1.4.1 Type I restriction enzymes:

- These enzymes have both restriction and modification activities. Restriction depends upon the methylation status of the target DNA.
- Cleavage occurs approximately 1000 bp away from the recognition site.
- The recognition site is asymmetrical and is composed of two specific portions in which one portion contain 3–4 nucleotides while another portion contain 4–5nucleotides and both the parts are separated by a non-specific spacer of about 6–8 nucleotides.
- They require S-adenosylmethionine (SAM), ATP, and magnesium ions (Mg^{2+}) for activity.

• These enzymes are composed of mainly three subunits, a specificity subunit that determines the DNA recognition site, a restriction subunit, and a modification subunit

2-1.4.2 Type II restriction enzymes:

- Restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification. Although the two enzymes recognize the same target sequence, they can be purified separately from each other.
- Cleavage of nucleotide sequence occurs at the restriction site.
- These enzymes are used to recognize rotationally symmetrical sequence which is often referred as palindromic sequence.
- These palindromic binding site may either be interrupted (e.g. BstEII recognizes the sequence 5´-GGTNACC-3´, where N can be any nucleotide) or continuous (e.g. KpnI recognizes the sequence 5´-GGTACC-3´).
- They require only Mg2+ as a cofactor and ATP is not needed for their activity.
- Type II endonucleases are widely used for mapping and reconstructing DNA in vitro because they recognize specific sites and cleave just at these sites. The steps involved in DNA binding and cleavage by a type II restriction endonuclease:
- These enzymes have nonspecific contact with DNA and initially bind to DNA as dimmers.
- The target site is then located by a combination of linear diffusion or “sliding” of the enzyme along the DNA over short distances, and hopping/jumping over longer distances. Once the target restriction site is located, the recognition process (coupling) triggers large conformational changes of the enzyme and the DNA, which leads to activation of the catalytic centre.
- Catalysis results in hydrolysis of phosphodiester bond and product release.

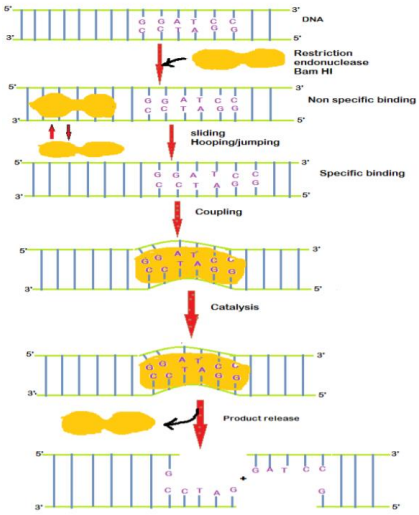


Fig 2-1.4.2: Structures of free, nonspecific, and specific DNA-bound forms of BamHI.

The two dimers are shown in brown, the DNA backbone is in green and the bases in grey. BamHI becomes progressively more closed around the DNA as it goes from the nonspecific to specific DNA binding mode.

2-1.4.3 Type III restriction enzymes:

- These enzymes recognize and methylate the same DNA sequence but cleave 24–26 bp away.
- They have two different subunits, in which one subunit (M) is responsible for recognition and modification of DNA sequence and another subunit (R) has nuclease action.
- Mg+2 ions, ATP are needed for DNA cleavage and process of cleavage is stimulated by SAM.
- Cleave only one strand. Two recognition sites in opposite orientation are necessary to break the DNA duplex.

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non- specific location > 1000 bp away from recognition site	Cut both strands at a specific, usually palindromic recognition site (4-8 bp)	Cleavage of one strand, only 24-26 bp downstream of the 3′ recognition site
Restriction and modification	Single multifunctional enzyme	Separate nuclease and methylase	Separate enzymes sharing a common subunit
Nuclease subunit structure	Heterotrimer	Homodimer	Heterodimer
Cofactors	ATP, Mg2+, SAM	Mg2+	Mg2+ (SAM)
DNA cleavage requirements	Two recognition sites in any orientation	Single recognition site	Two recognition sites in a head-to-head orientation
Enzymatic turnover	No	Yes	Yes
DNA translocation	Yes	No	No
Site of methylation	At recognition site	At recognition site	At recognition site

Table 2-1.4: Comparative properties of restriction enzymes

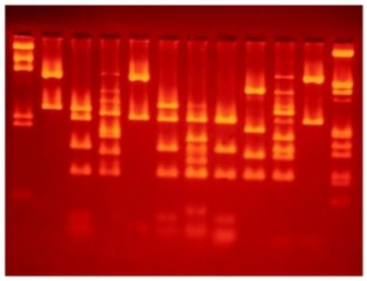


Fig 2-1.4.3: Cleaving a DNA sequence by a restriction enzyme creates a specific pattern .

Cleaving a single piece of DNA with multiple restriction enzymes creates a "DNA fingerprint." The pattern of fragments can be compared to similar DNA from another source treated with the same enzymes, to determine if the two are identical or different.

2-1.5 Cleavage Patterns of Some Common Restriction Endonucleases:

The recognition and cleavage sites and cleavage patterns of HindIII, SmaI, EcoRI, and BamHI are shown. Cleavage by an endonuclease creates DNA sequence with either a sticky end or blunt end. The blunt ended fragments can be joined to any other DNA fragment with blunt ends using linkers/adapters, making these enzymes useful for certain types of DNA cloning experiments.

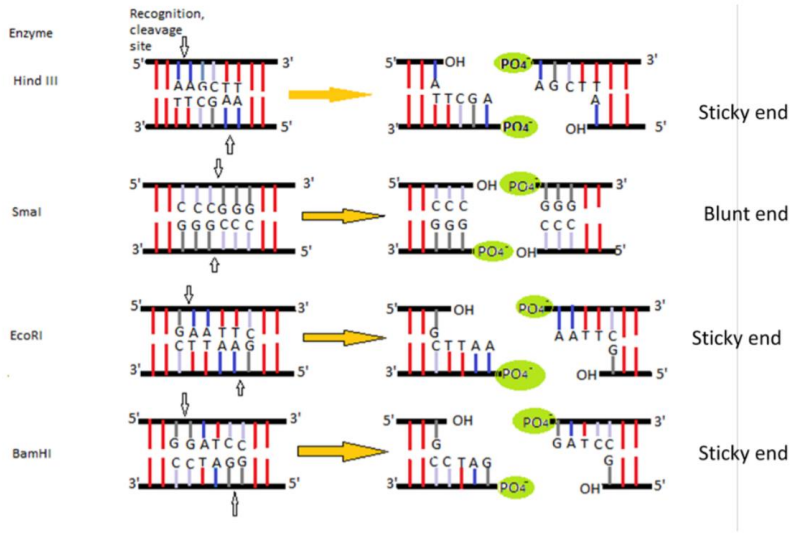


Fig 2-1.5: Cleavage patterns of *HindIII, SmaI, EcoRI* and *BamHI*

2-1.6 Applications:

In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.

- They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.
- Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restriction site present in the allele.
- Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.

ENZYMES IN MODIFICATION of DNA

2-2.1 Polynucleotide phosphorylase:

- Polynucleotide phosphorylase was first discovered from extracts of *Azotobacter agile* by Grunberg-Manago and Ochoa.
- Polynucleotide phosphorylase (PNPase) catalyzes the synthesis of long chain polyribonucleotides (RNA) in 5’ to 3’ direction from nucleotide diphosphates as precursors and reversibly catalyzes phosphorolytic cleavage of polyribonucleotides in 3’ to 5’ direction with a release of orthophosphate in presence of inorganic phosphate.
- PNPase is a bifunctional enzyme and functions in mRNA processing and degradation inside the cell.
- Structural and physiochemical studies in enzymes showed that it is formed of subunits. The arrangements of the subunits may vary from species to species which would alter their properties.
- These enzyme can catalyze not only the synthesis of RNA from the mixtures of naturally occurring ribonucleoside diphosphates, but also that of non-naturally occurring polyribonucleotides

2-2.1.1 Mechanism of action: As mentioned earlier, polynucleotide phosphorylase is a bifunctional enzyme. The mechanism of action of this enzyme can be represented by following reactions:

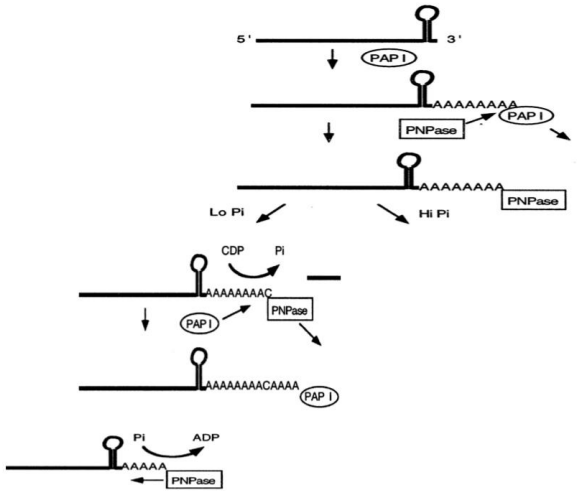


Fig 2-2.1: Schematic representation of the role of PNPase in poly(A) tail metabolism in *E. coli*.

(Source: Mohanty B K, and Kushner S R PNAS 2000; 97:11966-11971, copyright 2000, National Academy of Science*)*

In E.coli, polynucleotide phosphorylase regulates mRNA processing either by adding ribonucleotides to the 3' end or by cleaving bases in 3' to 5' direction. The function of PNPase depends upon inorganic phosphate (Pi) concentration inside the cell. The transcripts are polyadenylated using enzyme polyadenylate polymerase I (PAPI). After primary polyadenylylation of the transcript by PAP I, PNPase may bind to the 3′ end of the poly(A) tail. PNPase works either degradatively or biosynthetically inside the cell depending on the Pi concentration. Under high Pi concentration, it degrades the poly(A) tail releasing adenine diphosphates. If the Pi concentration is low, PAP Iinitiates addition of one or more nucleotides to the existing poly (A) tail and in the process generates inorganic phosphate. On dissociation of PNPase, the 3′ end again is available to PAP I for further polymerization.

2-2.1.2 Function: Different functions of Polynucleotide phosphorylase are:

• It is involved in mRNA processing and degradation in bacteria, plants, and in humans.

• It synthesizes long, highly heteropolymeric tails in vivo as well as accounts for all of the observed residual polyadenylation in poly(A) polymerase I deficient strains.

• PNPase function as a part of RNA degradosome in *E.coli* cell. RNA degradosome is a multicomponent enzyme complex that includes RNaseE (endoribinuclease), polynucleotide phosphorylase (3' to 5' exonuclease), RhlB helicase (a DEAD box helicase) and a glycolytic enzyme enolase. This complex catalyzes 3' to 5' exonuclease activity in presence of ATP. In eukaryotes, the exosomes are located in nucleus and cytoplasm. Degradsomes in bacteria and exosomes in eukaryotes are associated with processing, control and turnover of RNA transcripts.

• In rDNA cloning technology, it has been used to synthesize radiolabelled polyribonucleotides from nucleoside diphosphate monomers.

2-2.2 Deoxyribonuclease (DNase):

• A nuclease enzyme that can catalyze the hydrolytic cleavage of phosphodiester bonds in the DNA backbone are known as deoxyribonuclease (DNase).

• Based on the position of action, these enzymes are broadly classified as endodeoxyribonuclease (cleave DNA sequence internally) and exodeoxyribonuclease (cleave the terminal nucleotides). Unlike restriction enzymes, DNase does not have any specific recognition/restriction site and cleave DNA sequence at random locations.

• There is a wide variety of deoxyribonucleases known which have different substrate specificities, chemical mechanisms, and biological functions. They are:

1) **Deoxyribonuclease I (DNaseI):**

An endonuclease which cleaves double-stranded DNA or single stranded DNA. The cleavage preferentially occurs adjacent to pyrimidine (C or T) residues. The major products are 5'-phosphorylated bi-, tri- and tetranucleotides. It requires divalent ions (Ca2+ and Mn2+/Mg2+) for its activity and creates blunt ends or 1-2 overhang sequences. DNaseI is the most widely used enzyme in cloning experiments to remove DNA contamination from mRNA preparation (to be used for cDNA library preparation, northern hybridization, RT-PCR etc). The mode of action of DNaseI varies according to the divalent cation used. In the presence of magnesium ions (Mg+2), DNaseI hydrolyzes each strand of duplex DNA producing single stranded nicks in the DNA backbone, generating various random cleavages. On the other hand, in the presence of manganese ions (Mn+2), DNaseI cleaves both strands of a double stranded DNA at approximately the same site, producing blunt ended DNA fragments or with 1-2 base overhangs. The two major DNases found in metazoans are: deoxyribonuclease I and deoxyribonuclease II.

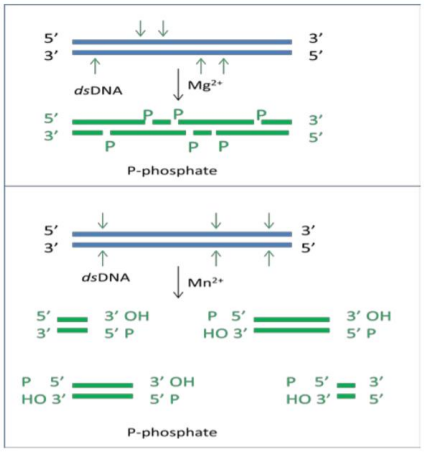


Fig 2-2.2 Action of DNase I in the presence of Mg^{+2} and Mn^{+2} ions. (Arrowhead denoting random site of cleavage in double stranded DNA by DNase I)

Some of the common applications of DNase I in rDNA technology have been mentioned below:

• Eliminating DNA contamination (e.g. plasmid) from preparations of RNA.

• Analyzing the DNA-protein interactions via DNA footprinting.

• Nicking DNA prior to radio-labeling by nick translation.

2) DeoxyribonucleaseII (DNaseII):

It is a non-specific endonuclease with optimal activity at acidic pH (4.5-5.5) and conserved from human to C.elegans.. It does not require any divalent cation for its activity. DNaseII initially introduces multiple single stranded nicks in DNA backbone and finally generates 3' phosphate groups by hydrolyzing phosphodiester linkages. This enzyme releases 3'phosphate groups by hydrolyzing phosphodiester linkage and creating nicks in the DNA backbone. DNaseII acts by generating multiple single stranded nicks followed by production of acid soluble nucleotides and oligonucleotides. The catalytic site of the enzyme contains three histidine residues which are essential for enzyme activity.

Some of the common applications of DNase II are as follows:

• DNA fragmentation

• Molecular weight marker

• Cell apoptosis assays etc.

3) Exonuclease III:

Exonuclease III is a globular enzyme which has 3'→5' exonuclease activity in a double stranded DNA. The template DNA should be double stranded and the enzyme does not cleave single stranded DNA. The enzyme shows optimal activity with blunt ended sequences or sequences with 5' overhang. Exonuclease III enzyme has a bound divalent cation which is essential for enzyme activity. The mechanism of the enzyme can be affected by variation in temperature, monovalent ion concentration in the reaction buffer, and structure and concentration of 3'termini. The enzyme shows optimal activity at 37°C at pH 8.0.

Various application of exonuclease III in molecular cloning experiments are:

• To generate template for DNA sequencing

• To generate substrate for DNA labeling experiments

• Directed mutagenesis

• DNA-protein interaction assays (to find blockage of exonuclease III activity by protein-DNA binding) etc.

4) Mung bean nuclease:

As the name suggest, this nuclease enzyme is isolated from mung bean sprouts (Vigna radiata). Mung bean nuclease enzymes can degrade single stranded DNA as well RNA. Under high enzyme concentration, they can degrade double stranded DNA, RNA or even DNA/RNA hybrids. Mung bean nuclease can cleave single stranded DNA or RNA to produce 5'-phosphoryl mono and oligonucleotides. It requires Zn2+ ion for its activity and shows optimal activity at 37°C. The enzyme works in low salt concentration (25mM ammonium acetate) and acidic pH (pH 5.0). Treatment with EDTA or SDS results in irreversible inactivation of the enzyme. Mung bean nuclease is less robust than S1 nuclease and easier to handle. It has been used to create blunt end DNA by cleaving protruding ends from 5' ends. This enzyme cannot produce nicks in a double stranded DNA but at higher concentration, it can generate nicks and cleave double stranded DNA.

ENZYMES IN MODIFICATION- PHOSPHATASES AND METHYLASES AND THEIR MECHANISM OF ACTION

2-3.1 Phosphatase:

- Phosphatase catalyses the cleavage of a phosphate (PO4 -2) group from substrate by using a water molecule (hydrolytic cleavage).
- This reaction is not reversible.
- This shows totally opposite activity from enzyme like kinase and phosphorylase that add a phosphate group to their substrate. On the basis of their activity there are two types of phosphatase i.e acid phosphatase and alkaline phosphatase. In both forms the alkaline phosphatase are most common.
- Special class of phosphatase that remove a phosphate group from protein, called "Phosphoprotein phosphatase".

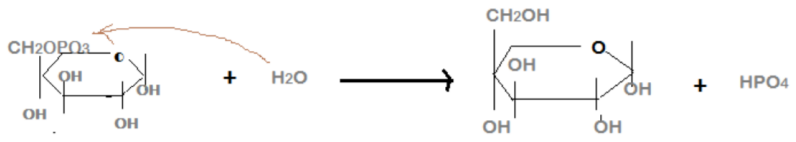


Fig 2-3.1: Schematic representation of hydrolytic cleavage of phosphate group (-PO_4^{-2}).

2-3.1.1 Acid phosphatase:

• It shows its optimal activity at pH between 3 and 6, e.g. a lysosomal enzyme that hydrolyze organic phosphates liberating one or more phosphate groups. They are found in prostatic epithelial cells, erythrocyte, prostatic tissue, spleen, kidney etc. 2-3.1.2 Alkaline phosphatase:

• Homodimeric enzyme which catalyzes reactions like hydrolysis and transphosphophorylation of phosphate monoester. • They show their optimal activity at pH of about 10. Alkaline phosphatase was the first zinc enzyme

discovered having three closed spaced metal ion. Two Zn+2 ions and one Mg+2 ion, in which Zn+2 ions are bridges by Asp 51. The mechanism of action is based on reaction where a covalent serine – phosphate intermediate is formed to produce inorganic phosphate and an alcohol.

• In human body it is present in four isoforms, in which three are tissue specific isoform i.e. placental, germ cell, intestinal and one is non tissue specific isoform. The genes that encode for tissue specific isoforms are present on chromosome -2 p37-q37, while the genes for one non tissue specific are present on chromosome - 1 p34- p36.1.

• During post-translational modification, alkaline phosphatase is modified by N-glycosylation. It undergoes a modification through which uptake of two Zn+2 ion and one Mg+2 ion occurs which is important in forming active site of that enzyme. Alkaline phosphatases are isolated from various sources like microorganisms, tissue of different organs, connective tissue of invertebrate and vertebrate, and human body.

Fig 2-3.1.2: Action of alkaline phosphatase

There are several AP that are used in gene manipulation- •

Bacterial alkaline phosphatase (BAP) - Bacterial alkaline phosphate is a phosphomonoester that hydrolyzes 3' and 5' phosphate from nucleic acid (DNA/ RNA). It more suitably removes phosphate group before end labeling and remove phosphate from vector prior to insert ligation. BAP generally shows optimum activity at temperature 65°C. BAP is sensitive to inorganic phosphate so in presence of inorganic phosphates activity may reduce. Two primary uses for alkaline phosphatase in DNA modification:

• Removing 5' phosphate from different vector like plasmid, bacteriophage after treating with restriction enzyme. This treatment prevents self ligation because unavailability of phosphate group at end. So, this treatment greatly enhances the ligation of desired insert. During ligation of desired insert, the complementary ends of the insert and vector will come to proximity of each other (only for sticky ends but not for blunt ends). One strand of the insert having 5'-phosphate will ligate with the 3'OH of the vector and the remaining strand will have a nick. This nick will be sealed in the next step by ligase enzyme in the presence of ATP. It is used to remove 5' phosphate from fragment of DNA prior to labelling with radioactive phosphate.

2-3.2 Methylase:

- Methyltransferase or methylase catalyzes the transfer of methyl group (-CH3) to its substrate. The process of transfer of methyl group to its substrate is called methylation.
- Methylation is a common phenomenon in DNA and protein structure.
- Methyltransferase uses a reactive methyl group that is bound to sulphur in Sadenosyl methionine (SAM) which acts as the methyl donor.
- Methylation normally occurs on cytosine (C) residue in DNA sequence. In protein, methylation occurs on nitrogen atom either on N-terminus or on the side chain of protein.
- DNA methylation regulates gene or silence gene without changing DNA sequences, as a part of epigenetic regulation.
- In bacterial system, methylation plays a major role in preventing their genome from degradation by restriction enzymes. It is a part of restriction – modification system in bacteria.

Methyltransferase can be classified in three groups:

- m6A-generates N6 methyl adenosine,
- m4C-generates N4 methyl cytosine,
- m5C-generatesN5 methyl cytosine.

m6A and m4C methyltransferase are primarily found in prokaryotes. These enzymes are responsible for methylation of DNA sequences in order to prevent the host from digesting its own genome via its restriction enzyme.

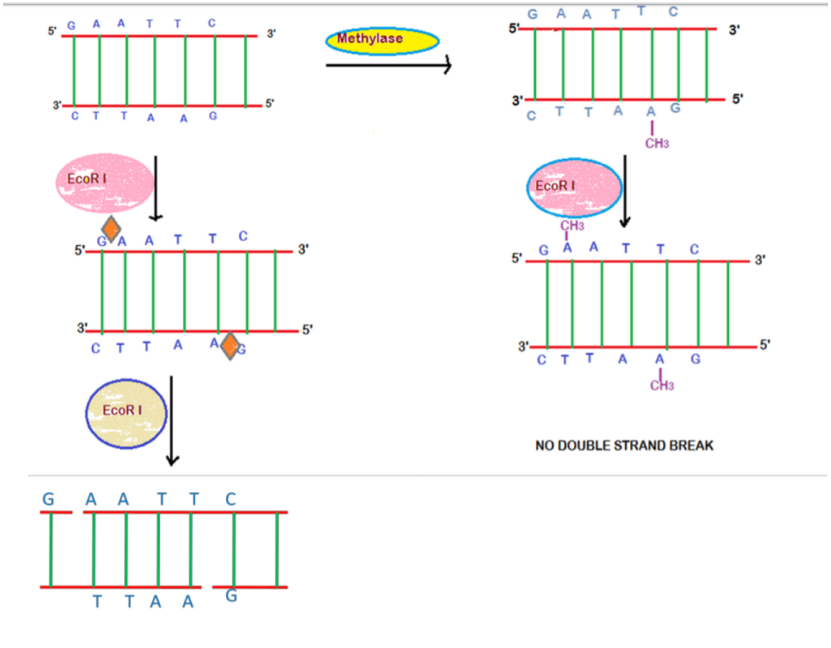


Fig 2-3.2: Activity of restriction and methylase enzymes

Restriction enzyme EcoRI cleaves within the recognition sequence if the DNA is unmethylated. On methylation by methylases, the restriction enzyme EcoRI is inhibited from cleaving within the restriction site. Some common examples of methytransferases are: DNA adenyl methytransferase (DAM), histone methyltransferase, O-methyltransferase etc. DAM methylase is generally used in recombinant DNA technology which can methylate adenine (A) in the sequence 5'GATC3'. This enzyme can methylate a newly synthesized DNA strand on specific sites.

ENZYMES IN MODIFICATION- LIGASES, POLYNUCLEOTIDE KINASE, RNASE AND THEIR MECHANISM OF ACTION

2-4.1 Ligases:

• DNA ligase catalyses the formation of phosphodiester bond between two deoxynucleotide residues of two DNA strands.

• DNA ligase enzyme requires a free hydroxyl group at the 3´ -end of one DNA chain and a phosphate group at the 5´-end of the other and requires energy in the process.

• *E.coli* and other bacterial DNA ligase utilizes NAD+ as energy donor, whereas in T4 bacteriophage, T4 DNA ligase uses ATP as cofactor.

• The role of DNA ligase is to seal nicks in the backbone of double-stranded DNA after lagging strand formation to join the okazaki fragments.

• This joining process is essential for the normal synthesis of DNA and for repairing damaged DNA. It has been exploited by genetic engineers to join DNA chains to form recombinant DNA molecules. Usually single stranded break is repaired using the complimentary strand as the template but sometimes double stranded breaks can also be repaired with the help of DNA ligase IV.

• The most widely used DNA ligase is isolated from T4 bacteriophage. T4 DNA ligase needs ATP as a cofactor. The enzyme from E. coli uses cofactor NAD.

2-4.1.1 Mechanism of Action of DNA Ligases:

- ATP, or NAD+, reacts with the ligase enzyme to form a covalent enzyme–AMP complex in which the AMP is linked to ε-amino group of a lysine residue in the active site of the enzyme through a phosphoamide bond.
- The AMP moiety activates the phosphate group at the 5´-end of the DNA molecule to be joined. It is called as the donor.
- The final step is a nucleophilic attack by the 3´-hydroxyl group on this activated phosphorus atom which acts as the acceptor. A phosphodiester bond is formed and AMP is released.
- The reaction is driven by the hydrolysis of the pyrophosphate released during the formation of the enzyme–adenylate complex. Two high-energy phosphate bonds are spent in forming a phosphodiester bond in the DNA backbone with ATP serving as energy source.
- The temperature optimum for T4 DNA ligase mediated ligation in vitro is 16˚C. However, ligation is also achieved by incubation at 4˚C by incubating over night or at room temperature condition by incubating for 30 minutes.
- Adenylate and DNA-adenylate are the important intermediates of the phosphodiester bond forming pathway.

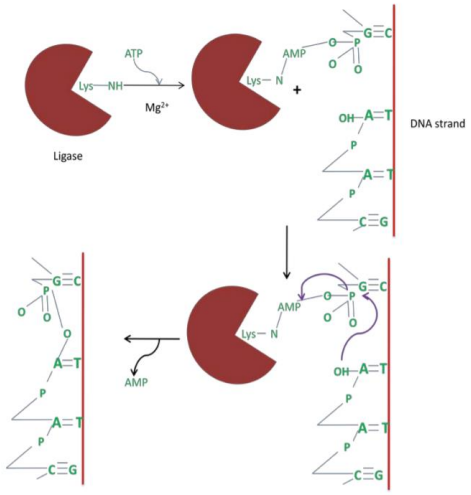


Fig 2-4.1.1: The mechanism of DNA joining by DNA ligase.

2-4.1.2 Application:

- DNA ligase enzyme is used by cells to join the “okazaki fragments” during DNA replication process. In molecular cloning, ligase enzyme has been routinely used to construct a recombinant DNA. Followings are some of the examples of application of ligase enzyme in molecular cloning.Joining of adapters and linkers to blunt end DNA molecule.
- Cloning of restricted DNA to vector to construct recombinant vector.

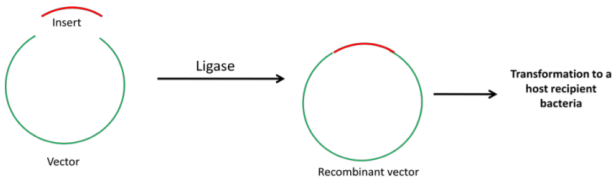


Fig 2-4.1.2: Ligation of a gene fragment into the vector and transformation of the cell.

2-4.2 Polynucleotide Kinase:

- PNK is a homotetramer with phosphatase activity at 3’ end and kinase activity at 5’ end with a tunnel like active site. The active site has side chains which interact with NTP donor’s beta-phosphate and 3’ phosphate of acceptor with an acid which activated 5’ –OH. Lys-15 and Ser-16 are important for the kinase activity of the enzyme.
- The basic residues of active site of PNK interact with the negatively charged phosphates of the DNA.

- Polynucleotide kinase (PNK) catalyzes the transfer of a phosphate group (PO4 -2) from γ position of ATP to the 5' end of either DNA or RNA and nucleoside monophosphate. PNK can convert 3' PO4/5' OH ends into 3' PO4/5' PO4 ends which blocks further ligation by ligase enzyme.
- PNK is used to label the ends of DNA or RNA with radioactive phosphate group.
- T4 polynucleotide kinase is the most widely used PNK in molecular cloning experiments, which was isolated from T4 bacteriophage infected E.coli.

2-4.3 Ribonuclease (RNase):

- Nuclease that can catalyze hydrolysis of ribonucleotides from either single stranded or double stranded RNA sequence are called ribonucleotides (RNase).
- RNase are classified into two types depending on position of cleavage, i.e. endoribonuclease (cleave internal bond) and exoribonuclease (cleave terminal bond).
- RNase is important for RNA maturation and processing.
- •RNase A and RNase H play important role in initial defence mechanism against RNA viral infection.

Two common types of ribonucleases are discussed below:

2-4.3.1 Ribonuclease A (RNase A):

- An endo-ribonuclease that cleaves specifically single-stranded RNA at the 3' end of pyrimidine residues.
- The RNA is degraded into 3'-phosphorylated mononucleotides C and U residues and oligonucleotides in the form of 2', 3'-cyclic monophosphate intermediates.
- Optimal temperature for RNase A is 60˚C (activity range 15-70˚C) and optimal pH is 7.6.
- RNase A has two histidine residues in its active site (His12 and His119). In the first step, His12 acts as a base; accepting proton forming a nucleophile which then attacks positively charged phosphorus atom. His119 acts as an acid in this case, donating a proton to oxygenated P-O-R' bond. The imidazole side chain acts as base in His 12 here. The side chain of Lys41 and Phe120 further stabilize the transition state. Nitrogen of the main chain of Phe120 donates hydrogen, thus bonding with the unbound oxygen atom.
- In the second step the acid base activities get reversed and His119 accepts proton from water causing hydroxyl attack on cyclic intermediate.
- Activity of RNase A can be inhibited by alkylation of His12 and His119 residue essential for activity of the enzyme. Application:
- It is used to remove RNA contamination from DNA sample.

2-4.3.2 Ribonuclease H:

- Non-specific endoribonuclease that degrades RNA by hydrolytic mechanism from DNA/RNA duplex resulting in single stranded DNA.
- Enzyme bound divalent metal ion is a cofactor here. The product formed is 5' phosphorylated ssDNA.
- During cDNA library preparation from RNA sample, RNaseH enzyme is used to cleave RNA strand of DNA-RNA duplex.

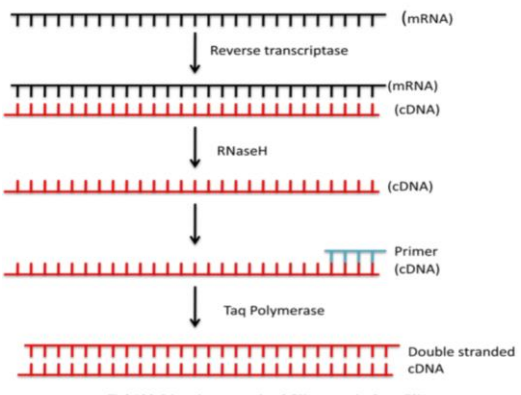
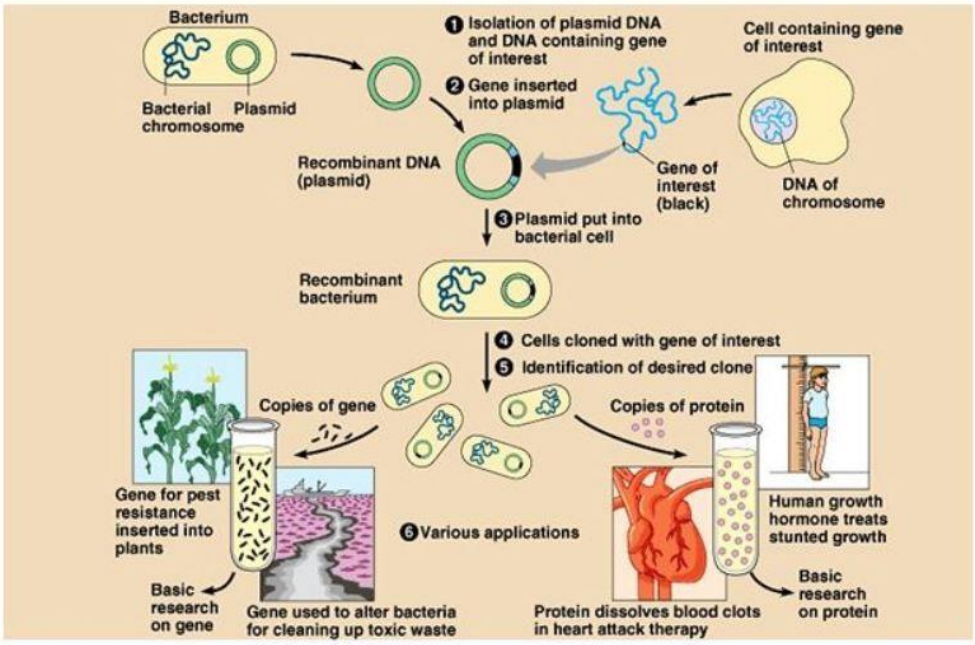


Fig 2-4.3.2: Schematic representation of cDNA preparation from mRNA

Cloning Vectors

A vector is a DNA molecule which is used for transporting exogenous DNA into the host cell. A vector is capable of self-replication and stable integration inside the host cell. The molecular analysis of DNA has been made possible only after the discovery of vectors. The whole process of molecular cloning involves the following steps:

1. Digestion of DNA fragments of the target segment and the vector DNA with the help of restriction enzymes,
2. Ligation of the target segment with the vector DNA with the help of DNA ligases, and
3. Introduction of the ligated segment into the host cell for propagation.



Vectors in Molecular Biology

General characteristics of a vector:

- It should have an Origin of Replication, known as ori, so that the vector is capable of autonomous replication inside the host organism.
- It must possess a compatible restriction site for insertion of DNA molecule.
- A vector should always harbour a selectable marker to screen the recombinant organism. This selectable marker can be an antibiotic resistance gene.
- For easy incorporation into the host machinery, a vector should itself be small in size and be able to integrate large size of the insert.

CLONING VECTOR

A cloning vector is also a fragment of DNA which is capable of self-replication and stable maintenance inside the host organism. It can be extracted from a virus, plasmid or cells of a higher organism. Most of the cloning vectors are genetically engineered. It is selected based upon the size and the kind of DNA segment to be cloned.

The cloning vectors must possess the following general characteristics:

- It should small in size.
- It must have an origin of replication.
- It must also be compatible with the host organism.
- It must possess a restriction site.
- The introduction of donor fragment must not intervene with the self-replicating property of the cloning vector.

- A selectable marker, possibly an antibiotic resistance gene, must be present to screen the recombinant cells.
- It should be capable of working under the prokaryotic as well as the eukaryotic system.
- Multiple cloning sites should be present.

Importance of Cloning Vectors

Cloning Vectors are used as the vehicle for transporting foreign genetic material into another cell. This foreign segment of DNA is replicated and expressed using the machinery of the host organism. A cloning vector facilitates amplification of a single copy DNA molecule into many copies. Molecular gene cloning is difficult without the use of the cloning vectors.

History of Cloning Vectors

Herbert Boyer, Keiichi Itakura, and Arthur Riggs were three scientists working in the Boyer's lab, University of California, where they recognized a general cloning vector. This cloning vector had restriction sites for cloning foreign DNA and also, the expression of antibiotic resistance genes for the screening of recombinant/ transformed cells. The first vector used for cloning purposes was pBR322, a plasmid. It was small in size, nearly 4kB, and had two selectable markers.

Features of Cloning Vectors

1. Origin of Replication (ori)

- A specific set/ sequence of nucleotides where replication initiates.
- For autonomous replication inside the host cell.
- Foreign DNA attached to ori also begins to replicate.

2. Cloning Site

- Point of entry or analysis for genetic engineering.
- Vector DNA at this site is digested and foreign DNA is inserted with the aid of restriction enzymes.
- Recent works have discovered plasmids with multiple cloning sites (MCS) which harbour up to 20 restriction sites.

3. Selectable Marker

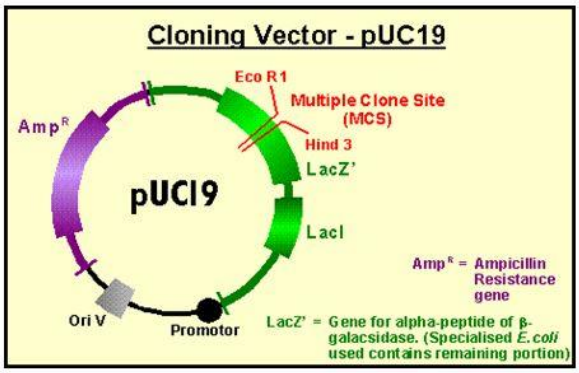
- Gene that confers resistance to particular antibiotics or selective agent which, under normal conditions, is fatal for the host organism.
- Confers the host cell the property to survive and propagate in culture medium containing the particular antibiotics.

4. Marker or Reporter Gene

- Permits the screening of successful clones or recombinant cells.
- Utilised extensively in blue-white selection.

5. Inability to Transfer via Conjugation

- Vectors must not enable recombinant DNA to escape to the natural population of bacterial cells.



Cloning vector

Types of Cloning Vectors

A. Plasmids

B. Bacteriophage

C. Phagemids

D. Cosmids

E. Bacterial Artificial Chromosome (BAC)

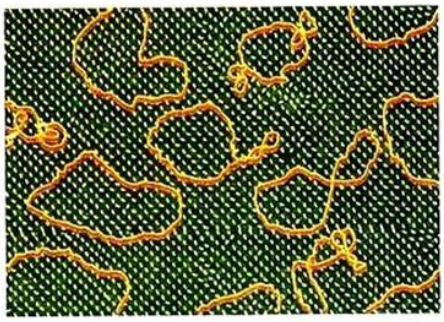
F. Yeast Artificial Chromosome (YAC)

G. Human Artificial Chromosome (HAC)

H. Retroviral Vectors

A. Plasmids

- Plasmids were the first vectors to be used in gene cloning.
- They are naturally occurring and autonomously replicating extra-chromosomal double-stranded circular DNA molecules. However, not all plasmids are circular in origin.



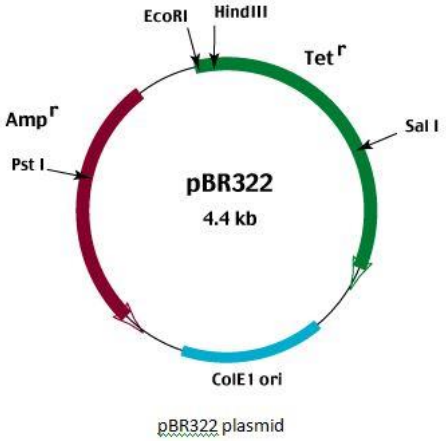
Electron micrograph of circular plasmid molecules isolated from E. coli

- They are present in bacteria, archaea, and eukaryotes.
- The size of plasmids ranges from 1.0 kb to 250 kb.
- DNA insert of up to 10 kb can be cloned in the plasmids.
- The plasmids have high copy number which is useful for production of greater yield of recombinant plasmid for subsequent experiments.
- The low copy number plasmids are exploited under certain conditions like the cloned gene produces the protein which is toxic to the cells.
- Plasmids only encode those proteins which are essential for their own replication. These protein-encoding genes are located near the ori.

Examples: pBR322, pUC18, F plasmid, Col plasmid.

Nomenclature of plasmid cloning vector: pBR322 cloning vector has the following elements:

- p= plasmid
- B= Bolivar (name of the scientist)
- R= Rodriguez (name of the scientist)
- 322= number of plasmid discovered in the same lab



pBR322 plasmid

Advantages of using Plasmids as vectors:

- Easy to manipulate and isolate because of small size.
- More stable because of circular configuration.
- Replicate independent of the host.
- High copy number.
- Detection easy because of antibiotic-resistant genes.

Disadvantages of using Plasmids as vectors:

- Large fragments cannot be cloned.
- Size range is only 0 to 10kb.
- Standard methods of transformation are inefficient.

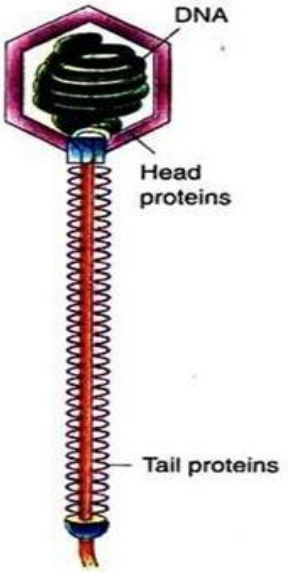
B. Bacteriophage

- Bacteriophages or phages are viruses which infect bacterial cells.
- The most common bacteriophages utilized in gene cloning are Phage λ and M13 Phage.
- A maximum of 53 kb DNA can be packaged into the phage.
- If the vector DNA is too small, it cannot be packaged properly into the phage.

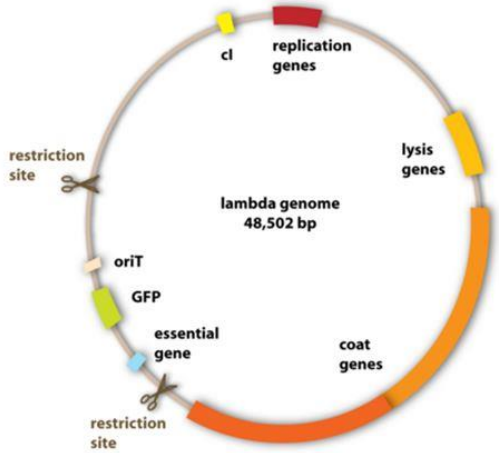
Examples: Phage Lambda, M13 Phage, etc.

Phage Lambda λ

- It has head, tail, and tail fibers.
- Its genome consists of 48.5 kb of DNA and 12 bp ss DNA which comprise of sticky ends at both the terminals. Since these ends are complementary, they are cohesive and also referred to as cos sites.
- Infection by λ phage requires adsorption of tail fibers on the cell surface, contraction of the tail, and injection of the DNA inside the cell.



Structure of bacteriophage λ



Bacteriophage λ

M13 Phage

- These vectors are used for obtaining single-stranded copies of the cloned DNA.
- They are utilized in DNA sequencing and in vitro mutagenesis.
- M13 phages are derived from filamentous bacteriophage M13. The genome of M13 is 6.4 kb.
- DNA inserts of large sizes can be cloned.
- From the double-stranded inserts, pure single-stranded DNA copies are obtained.

Types of Phage Vectors

There are 2 types of phage vectors:

1. **Insertion vectors-** these contain a particular cleavage site where the foreign DNA of up to 5-11 kb can be inserted.
2. **Replacement vectors-** the cleavage sites flank a region which contains genes not necessarily important for the host, and these genes can be deleted and replaced by the DNA insert.

Advantages of using Phage Vectors

- They are way more efficient than plasmids for cloning large inserts.
- Screening of phage plaques is much easier than identification of recombinant bacterial colonies.

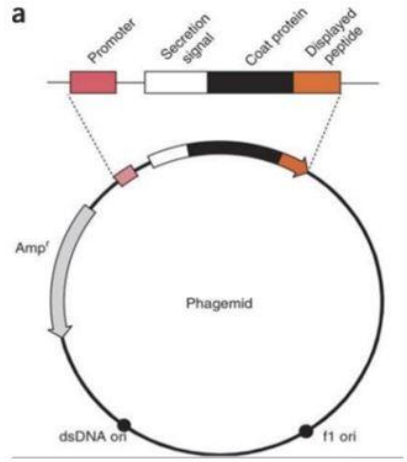
C. Phagemids or Phasmid

- They are prepared artificially.

- Phasmid contains the F1 origin of replication from F1 phage.
- They are generally used as a cloning vector in combination with M13 phage.
- It replicates as a plasmid and gets packaged in the form of single-stranded DNA in viral particles.

Advantages of using Phagemids:

- They contain multiple cloning sites.
- An inducible lac gene promoter is present.
- Blue-white colony selection is observed.



Phagemid

D. Cosmids

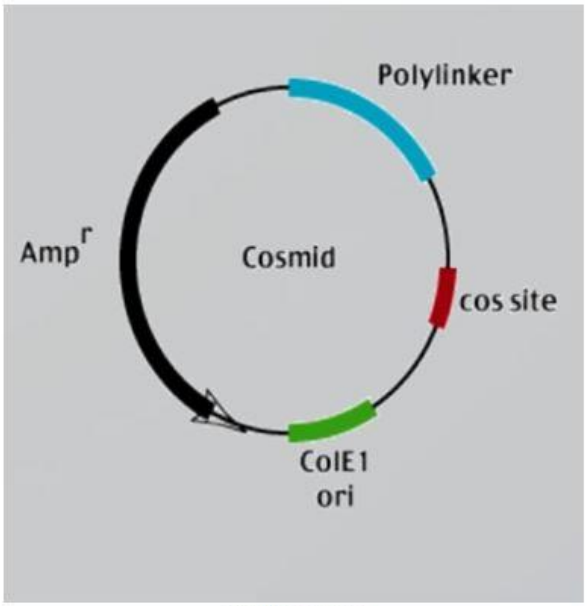
- Cosmids are plasmids.
- They are capable of incorporating the bacteriophage λ DNA segment. This DNA segment contains cohesive terminal sites (cos sites).
- Cos sites are necessary for efficient packaging of DNA into λ phage particles.
- Large DNA fragments of size varying from 25 to 45 kb can be cloned.
- They are also packaged into λ This permits the foreign DNA fragment or genes to be introduced into the host organism by the mechanism of transduction.

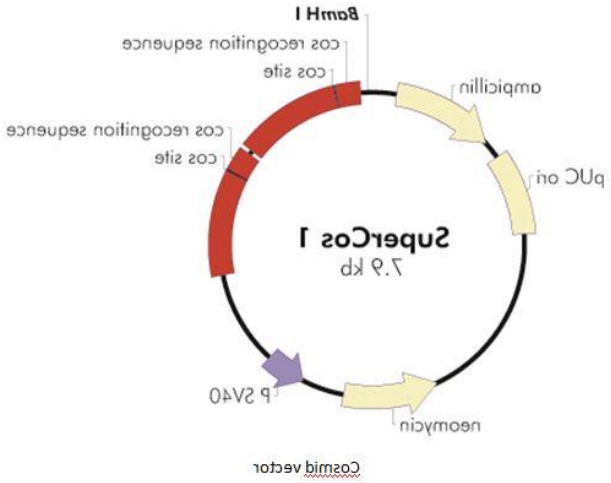
Advantages of using cosmids as vectors:

- They have high transformation efficiency and are capable of producing a large number of clones from a small quantity of DNA.
- Also, they can carry up to 45 kb of insert compared to 25 kb carried by plasmids and λ.

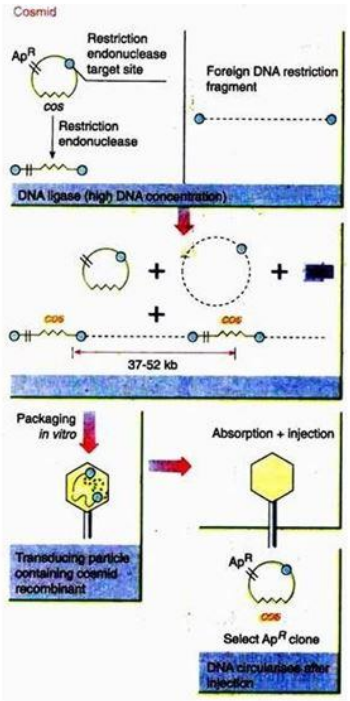
Disadvantages of using cosmids as vectors:

- Cosmids cannot accept more than 50 kb of the insert.





Cosmid vector



Cloning a Cosmid Vector

E. Bacterial Artificial Chromosomes (BACs)

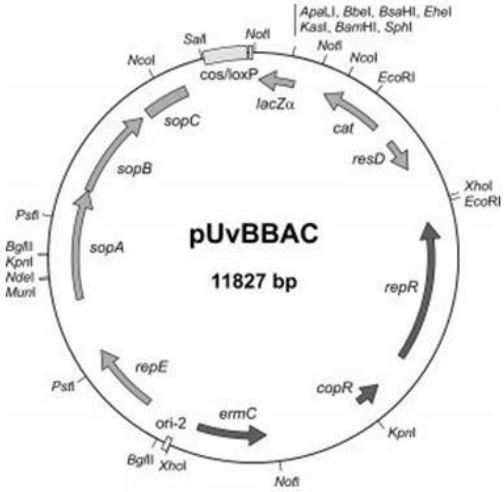
- Bacterial artificial chromosomes are similar to E. coli plasmid vectors.
- They contain ori and genes which encode ori binding proteins. These proteins are critical for BAC replication.
- It is derived from naturally occurring F' plasmid.
- The DNA insert size varies between 150 to 350 kb.

Advantages of BACs:

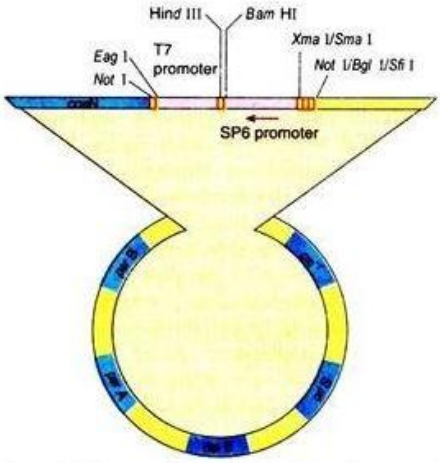
- They are capable of accommodating large sequences without any risk of rearrangement.
- BACs are frequently used for studies of genetic or infectious disorders.
- High yield of DNA clones is obtained.

Disadvantages of BACs:

- They are present in low copy number.
- The eukaryotic DNA inserts with repetitive sequences are structurally unstable in BACs often resulting in deletion or rearrangement.



Bacterial Artificial Chromosome



Structure of a BAC vector derived from a mini-F plasmid. Its *Ori*S and *rep*E genes mediate the unidirectional replication of the F factor, while *par* A and *par* B maintain the copy number at a level of one or two per genome. Cm^r is the chloramphenicol resistance gene, cosN and *lox*P are the cleavage sites for λ terminase and P1 *cre* protein. *Hind*III and *Bam*HI are cleavage sites for inserting foreign DNA

Bacterial Artificial Chromosome

F. Yeast Artificial Chromosomes (YACs)

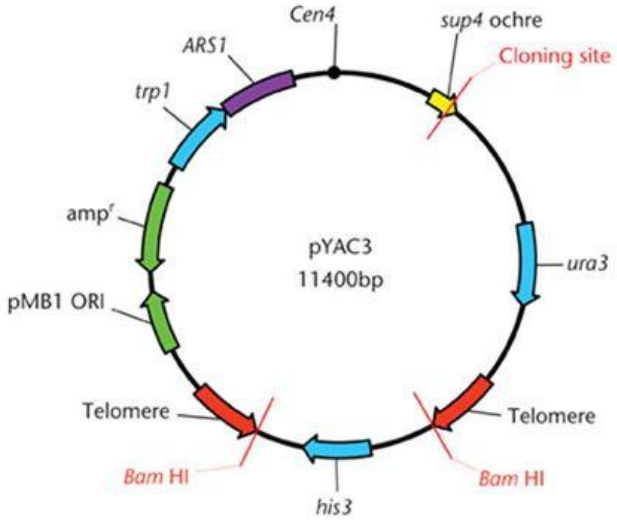
- A large DNA insert of up to 200 kb can be cloned.
- They are used for cloning inside eukaryotic cells. These act as eukaryotic chromosomes inside the host eukaryotic cell.
- It possesses the yeast telomere at each end.
- A yeast centromere sequence (CEN) is present which allows proper segregation during meiosis.
- The ori is bacterial in origin.
- Both yeast and bacterial cells can be used as hosts.

Advantages of using YACs:

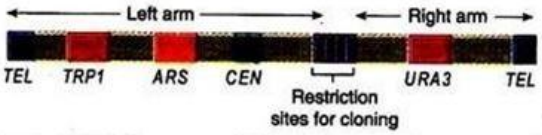
- A large amount of DNA can be cloned.
- Physical maps of large genomes like the human genome can be constructed.

Disadvantages of using YACs:

- Overall transformation efficiency is low.
- The yield of cloned DNA is also low.



Yeast Artificial Chromosome



Structure of yeast artificial chromosome (YAC) cloning vector. It contains a telomere (TEL) at each end, a yeast centromere sequence (CEN), a yeast selectable marker for each arm (*TRP*1 and *URA*3), a sequence that allows autonomous replication in yeast (ARS) and restriction sites for cloning

Yeast Artificial Chromosome

Advantages of BACs over YACs

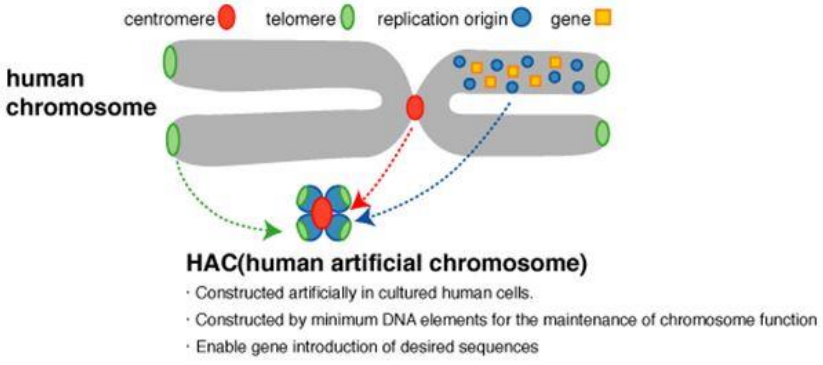
1. Comparatively stable.
2. Easy to transform.
3. Simple purification required.
4. User- friendly.
5. Aid in the development of vaccines.

G. Human Artificial Chromosome (HACs)

- Human artificial chromosomes are artificially synthesized.
- They are utilized for gene transfer or gene delivery into human cells.
- It can carry large amounts of DNA inserts.
- They are used extensively in expression studies and determining the function of the human chromosomes.

Advantages of using HACs:

- No upper limit on DNA that can be cloned.
- it avoids the possibility of insertional mutagenesis.



Human Artificial Chromosomes

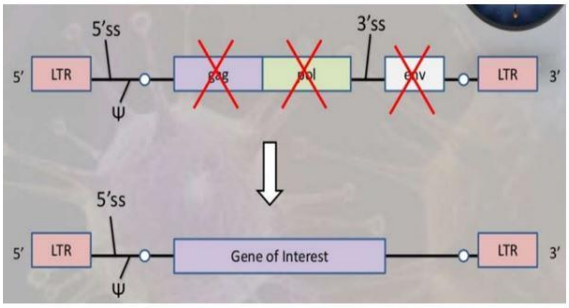
H. Retroviral Vectors

- Retroviruses are the virus with RNA as the genetic material.

- Retroviral vectors are used for introduction of novel or manipulated genes into the animal or human cells.
- The viral RNA is converted into DNA with the help of reverse transcriptase and henceforth, efficiently integrated into the host cell.
- Any gene of interest can be introduced into the retroviral genome. This gene of interest can then integrate into host cell chromosome and reside there.

Advantages of using retroviral vectors:

- They are widely used as a tool to study and analyze oncogenes and other human genes.



Retroviral vector

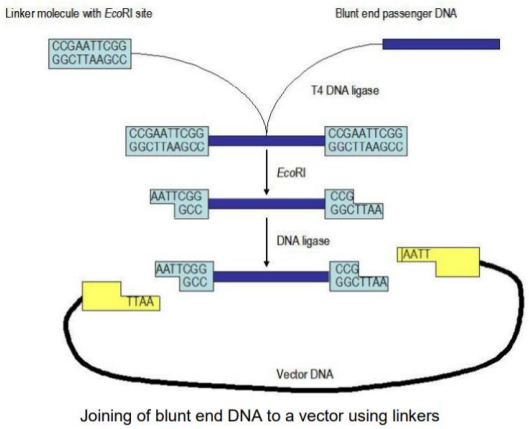
The things which matter while choosing a cloning vector are:

1. DNA insert size
2. Size of the vector
3. Restriction Size
4. Efficiency of cloning

Vector	Insert size (kb)
Plasmid	<10 kb
Bacteriophage	9 – 15 kb
Cosmids	23 – 45 kb
BACs	≤ 300 kb
PACs	100 – 300 kb
YACs	100 – 3000 kb

Linker:

The E. coli DNA ligase will not catalyze blunt end ligation except under special reaction conditions of macromolecular crowding. The unique property of T4 DNA ligase was used to ligate DNA fragments with blunt ends involving short decameric oligonucleotides called linkers. Using linkers Short oligonucleotides (decamers) which contain sites for one or more restriction enzymes are used to facilitate the ligation process among the DNA fragments with blunt ends.



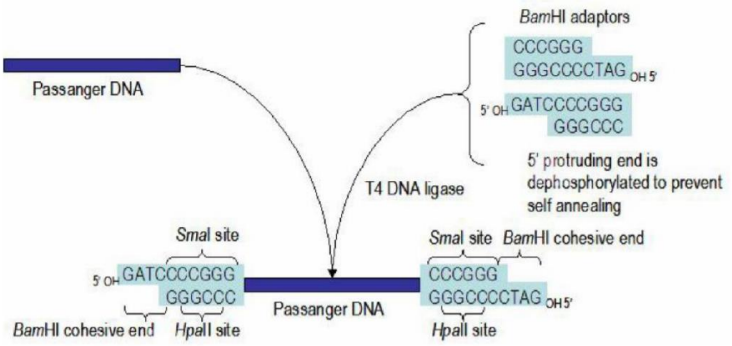
Joining of blunt end DNA to a vector using linkers

The linker molecules can be ligated to both ends of the foreign DNA to be cloned and then treated with restriction endonuclease to produce sticky end fragments which can be incorporated into a vector molecule that has been cut

with the same restriction endonuclease. Insertion by means of the linker creates restriction sites at each end of the foreign DNA, and thus enables the foreign DNA excised and recovered after cloning and amplification in the host bacterium.

Adaptors

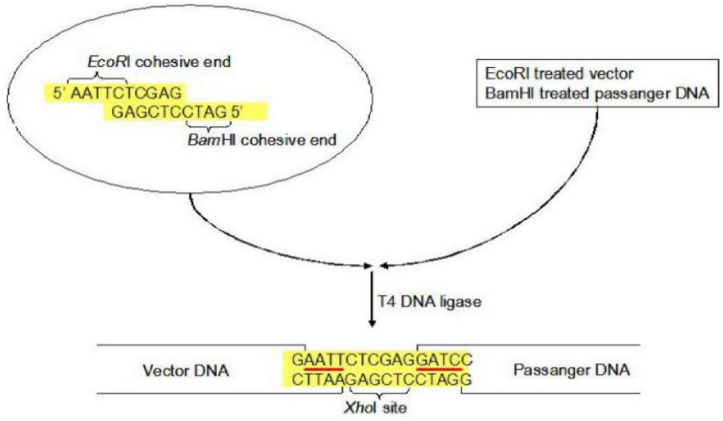
The other strategy adopted for ligating DNA fragments with blunt ends is using adaptors. The adaptor molecules are synthetic deoxynucleotides that can be used to join two incompatible cohesive ends, two blunt ends or a combination of both. Such adaptors are of several types viz., preformed, conversion and single stranded adaptors. Preformed adaptors Preformed adaptors are short DNA duplexes with at least one cohesive end. The problem of internal cleavage of the insert DNA can be overcome by using a preformed adaptor that will introduce a new restriction site. For example, an adaptor having BamHI cohesive ends and sites HpaII and SmaI can be attached to passenger DNA andinserted into a BamHI in vector. After cloning, passenger DNA can be excised from the hybrid by using any one of the enzymes that recognize the restriction sites within the adaptor region.



Use of preformed adaptors

Conversion adaptors

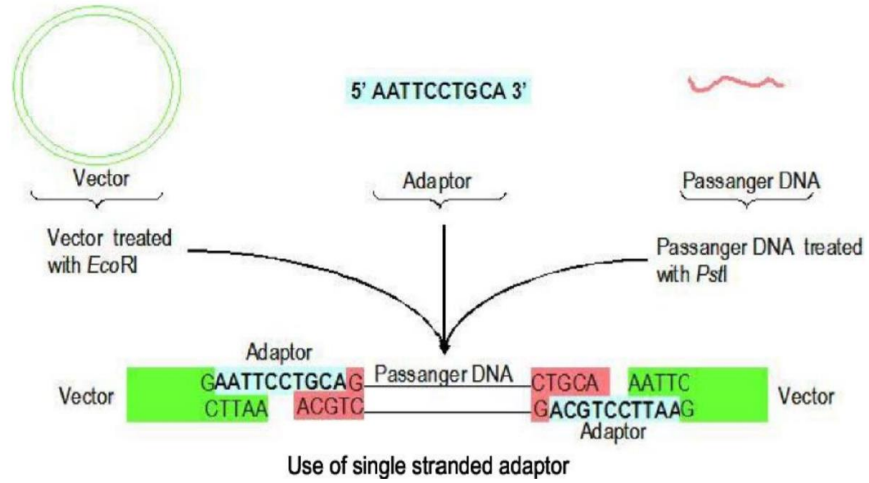
Conversion adaptors are synthetic oligonucleotides bearing different cohesive restriction termini. Such adaptors enable vector molecules that have been cleaved with one endonuclease to be joined to passenger fragments that have been cleaved with another. Often these adaptors contain internal restriction sites that permit recovery of the passenger fragment, for example, the EcoRI-BamHI adaptor contains a site for XhoI.



Use of conversion adaptor

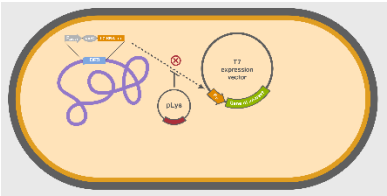
Single stranded adaptors

Single stranded adaptors can be used to make 3'-protruding cohesive ends compatible with 5' protruding ends. Such adaptors permit the insertion of passenger fragments into sites on vectors from which they would otherwise be precluded because of incompatible cohesive ends.



Expression vector

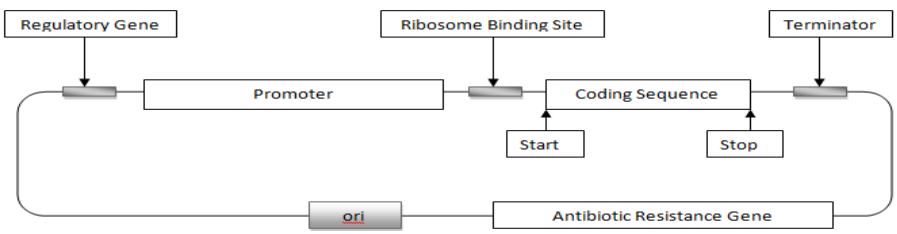
An **expression vector**, otherwise known as an **expression** construct, is usually a plasmid or virus designed for gene **expression** in cells. The **vector** is used to introduce a specific gene into a target cell, and can commandeer the cell's mechanism for protein synthesis to produce the protein encoded by the gene. The expression vectors are vectors which act as vehicles for DNA insert and also allow the DNA insert to be expressed efficiently. These may be plasmids or viruses. The expression vectors are also known as expression constructs.



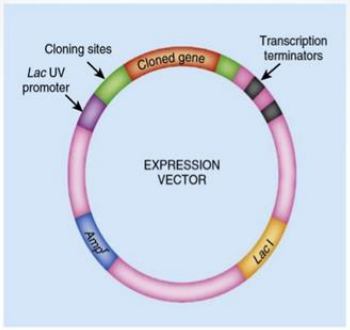
The expression vectors are genetically engineered for the introduction of genes into the target cells. In addition to the gene of interest, these expression constructs also contain regulatory elements like enhancers and promoters so that efficient transcription of the gene of interest occurs.

The simplest expression constructs are also known as transcription vectors; only because they allow transcription of the cloned foreign gene and not its translation. The vectors which facilitate both transcription and translation of the cloned foreign gene are known as protein expression vectors. These protein expression constructs also lead to the production of recombinant protein.

Now, for transcription and translation, a promoter and a termination sequence are a must. Transcription initiates at the promoter and ends at the termination site. The promoters of expression vectors must have on/off switches. These switches help in the regulation of production of the gene product. Excessive amounts of product of the gene of interest can be toxic for the cell. A common promoter utilized in the expression constructs is the mutant version of the lac promoter, lacUV. The lacUV promoter initiates a high level of transcription under induced conditions. Moreover, in some expression vectors, a ribosomal binding site is present upstream to the start codon. The ribosomal binding site facilitates the efficient translation of the cloned foreign gene.



Expression vectors are used extensively in molecular biology; in techniques like site-directed mutagenesis.



Expression Vectors Have Tightly Regulated Promoters: eg lacUV promoter. To stimulate transcription, the artificial inducer, IPTG, is added. IPTG binds to the LacI repressor protein, which then detaches from the DNA. This allows RNA polymerase to transcribe the gene. Before IPTG is added, the LacI repressor prevents expression of the cloned gene.

How do Expression Vectors work?

- Once the expression construct is inside the host cell, the protein encoded by the gene of interest is produced by the transcription. Thereafter, it utilizes the translation machinery and ribosomal complexes of the host organism.
- Frequently, the plasmid is genetically engineered to harbor regulatory elements like enhancers and promoters. These regulator sequences aid in efficient transcription of the gene of interest.
- Expression vectors are extensively used as tools which help in the production of mRNAs and, in turn, stable proteins. They are of much interest in biotechnology and molecular biology for the production of proteins like insulin. Insulin is the chief ingredient in the treatment of the complex disease, Diabetes.
- When the protein product is expressed, it is to be then purified. The purification of a protein poses a challenge since the protein of interest, whose gene is carried on the expression vector, is to be purified independently of the proteins of the host organism. To make the process of purification simpler, the gene of interest carried on the expression vector should always have a 'tag'. This tag can be any marker peptide or histidine (His tag).
- Expression vectors are considerably exploited in techniques like site-directed mutagenesis. Cloning vectors introduce the gene of interest into a plasmid which in turn replicates in bacteria. These cloning vectors need not necessarily result in the expression of a protein.

T**herefore, expression vectors must have the following expression signals:**

- Strong promoter,
- Strong termination codon,
- Adjustment of distance between the promoter and cloned gene,
- Inserted transcription termination sequence, and
- Portable translation initiation sequence.

Promoter

- A promoter ensures a reliable transcription of the gene of interest. Also, strong promoters are also necessary for an efficient mRNA synthesis with RNA polymerase.
- Regulation of the promoter is another critical aspect which should always be kept in mind while constructing an expression vector.
- The strongest promoters are those found in bacteriophages T5 and T7.

In *E. coli*, the promoter is regulated in two ways:
Induction: the addition of chemical switches on the transcription of the gene.
Repression: addition of chemical switches off the transcription of the gene.

The most commonly used promoters in *E. coli* expression system:

lac promoter:

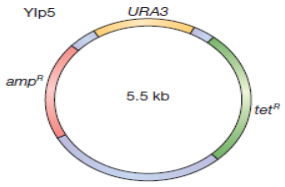
- It regulates the transcription of the lac Z gene. The lac Z gene is responsible for the production of β-galactosidase.
- The lac Z gene can be induced by IPTG, isopropylthiogalactosidase.

- The lac promoter sequences can be fused to the target gene. It will, then, result in lactose- dependent expression of the target gene.
- Nevertheless, the lac promoter has its drawbacks. It is quite weak and cannot be utilized for the high levels of production of the desired protein. In addition to this, the lac genes carry out the basal level of transcription even in the absence of induction (inducer molecule).

Expression using the T7 RNA polymerase/promoter system

Yeast Integrating plasmids (YIp): These plasmids lack an ORI and must be integrated directly into the host chromosome via homologous recombination. YIps (YIp5)

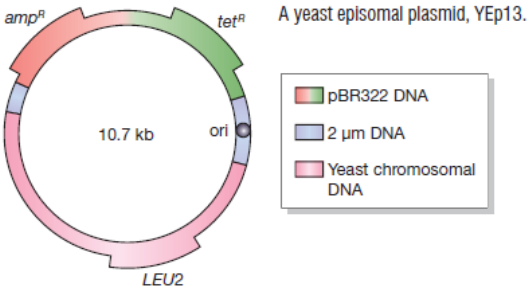
- They are the vectors having URA3 gene instead of LEU2. It has pBR322 plasmid.
- The size of the vector is 5.5 kb.
- URA3 synthesis for orotidine 5′-phosphate decarboxylase necessary for pyrimidine synthesis. Thus, we choose the auxotrophic mutant yeast cell which lacks the URA3 gene. Thus, only transformed yeast cell would culture.
- As the name suggests, this vector can integrate itself into the bacterial genome just like the YEps.
- The replication occurs when the vector is integrated into the bacterial system.
- The copy number is only 1.
- The transformation efficiency of the vector is less than 1000 cells per µg.



A YIp

Yeast Centromere plasmids (YCp): These are considered low copy vectors and incorporate part of an ARS along with part of a centromere sequence (CEN). These vectors replicate as though they are small independent chromosomes and are thus typically found as a single copy. Unlike the ARS vectors, CEN vectors are stable without integration.

Yeast Episomal plasmids (YEp): These are most similar to bacterial plasmids and are considered "high copy". A fragment from the 2 micron circle (a natural yeast plasmid) allows for 50+ copies to stably propogate per cell. The copy number of these vectors can also be controlled if specific regulatable elements are included

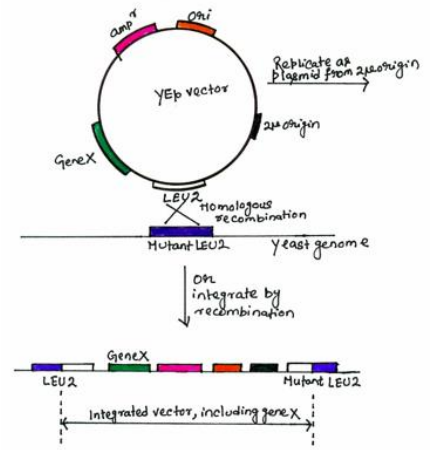


A yeast episomal plasmid, YEp13.

YEps were first constructed by Beggs (1978) by recombining an *E. coli* cloning vector with the naturally occurring yeast 2 µm plasmid. [3] The word "episomal" indicates that a YEp can replicate as an independent plasmid, but also implies that integration into one of the yeast chromosomes can occur. Integration occurs because the gene carried on

the vector as a selectable marker is very similar to the mutant version of the gene present in the yeast chromosomal DNA. [4]

This plasmid is 6.3 kb in size, has a copy number of 50–100 per haploid cell and has no known function. [3] YEps have the highest transformation frequency providing between 10,000 and 100,000 transformed cells per μg. [4]



Baculovirus.

The **baculovirus expression system** is commonly used to generate recombinant proteins in insect cells at high production levels. Recombinant **baculoviruses** can accommodate large segments of foreign DNA. A baculovirus expression vector (BEV) is a recombinant baculovirus with a double-stranded circular DNA genome that has been genetically modified to include a foreign gene of interest. BEVs are viable and can infect susceptible hosts, usually cultured lepidopteran insect cells or larvae, in a helperindependent fashion. Therefore, BEVs can efficiently transfer foreign genes into these eukaryotic host cells. The foreign gene is usually a chimeric construct with the sequence encoding a protein of interest placed under the transcriptional control of a viral promoter. This arrangement enables viral functions to transcribe the gene during infection. The resulting mRNA is translated and the newly synthesized protein modified by host-encoded biosynthetic machinery. In essence, then, BEVs and their insect cell hosts are two separate components of a binary eukaryotic expression system, which will be called simply the BEV system throughout the remainder of this chapter. The BEV system is among the best tools currently available for the expression of recombinant genes in a eukaryotic host. The BEV system has contributed immensely to basic research, since it has been used to produce hundreds of different recombinant proteins for further studies. This system also holds great promise for the industrial production of proteins with direct applications as vaccines, therapeutic agents, and/or diagnostic reagents. Fi nally, BEVs are being developed as improved biological pest control agents, as detailed in Chapter 13 (this volume). The most significant advantage of the BEV system over other expression systems is that it can be used to produce exceptionally large amounts of functional foreign proteins. The production levels provided by the BEV system are often comparable to those provided by prokaryotic systems, and at late times after infection, the recombinant protein usually constitutes a significant proportion of the total protein in the host cell. Unlike prokaryotic expression systems, however, the BEV system has eukaryotic protein processing capabilities, which enables it to produce more authentic foreign proteins. Thus, it is the potential of this system to provide prokaryotic levels of foreign gene expression in a eukaryotic background that makes it so powerful and attractive. Another advantage of this system is that BEVs are noninfectious for vertebrates, so they are relatively safe for laboratory manipulation and industrial production of pharmaceutically important proteins. Finally, the actual process of isolating BEVs has become increasingly fast, simple, and efficient as more sophisticated molecular tools have been developed. While it must be appreciated that the BEV system has been used with routine success to produce many different foreign gene proteins for over a decade, it also must be recognized that this system has some limitations that need to be addressed through further research and development. One limitation is that there is significant protein-to-protein variation in the production levels provided by this system. Generally, some classes of recombinant proteins, such as membrane-bound and secreted glycoproteins, are produced at much lower levels than others. Another limitation is that insect cell protein processing pathways are not necessarily identical to those of

higher eukaryotes, and as a result, covalent chemical modifications of recombinant proteins produced in this system may differ from those on the native protein. Finally, the BEV system provides only transient expression, because the foreign gene is delivered into the host cell by a virus that ultimately kills it. A related problem is that certain protein processing pathways are compromised by baculovirus infection, and this can decrease the efficiency and kinetics of recombinant protein processing well before the cell dies.

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