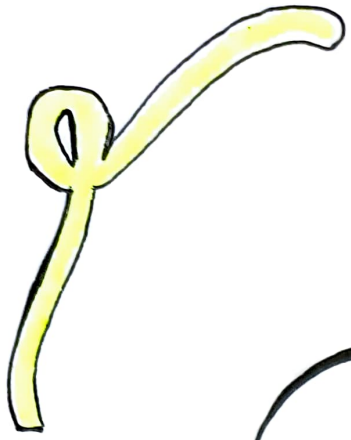

CLONING VECTORS

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Assignment (Cloning Vectors)

Cloning Vectors

An autonomously replicating genetic element used to carry a complementary DNA or fragments of genomic DNA into a host cell for the purpose of gene cloning. A viral DNA or plasmid can be used as a cloning vector.

Cloning Vectors for E. coli

1. Cloning vectors based on plasmid

Plasmids are naturally occurring circular, extrachromosomal, autonomously replicating DNA, present in many prokaryotic and few eukaryotic organisms. Plasmid vectors provide a simple way of cloning small DNA fragments in bacterial (and simple eukaryotic cells) cells. The ideal plasmid cloning vector must have small size, high copy number, own origin of replication, restriction sites for many restriction enzymes & selectable markers. Thus, in order to adapt natural plasmid molecules as cloning vectors, several modifications are normally made.

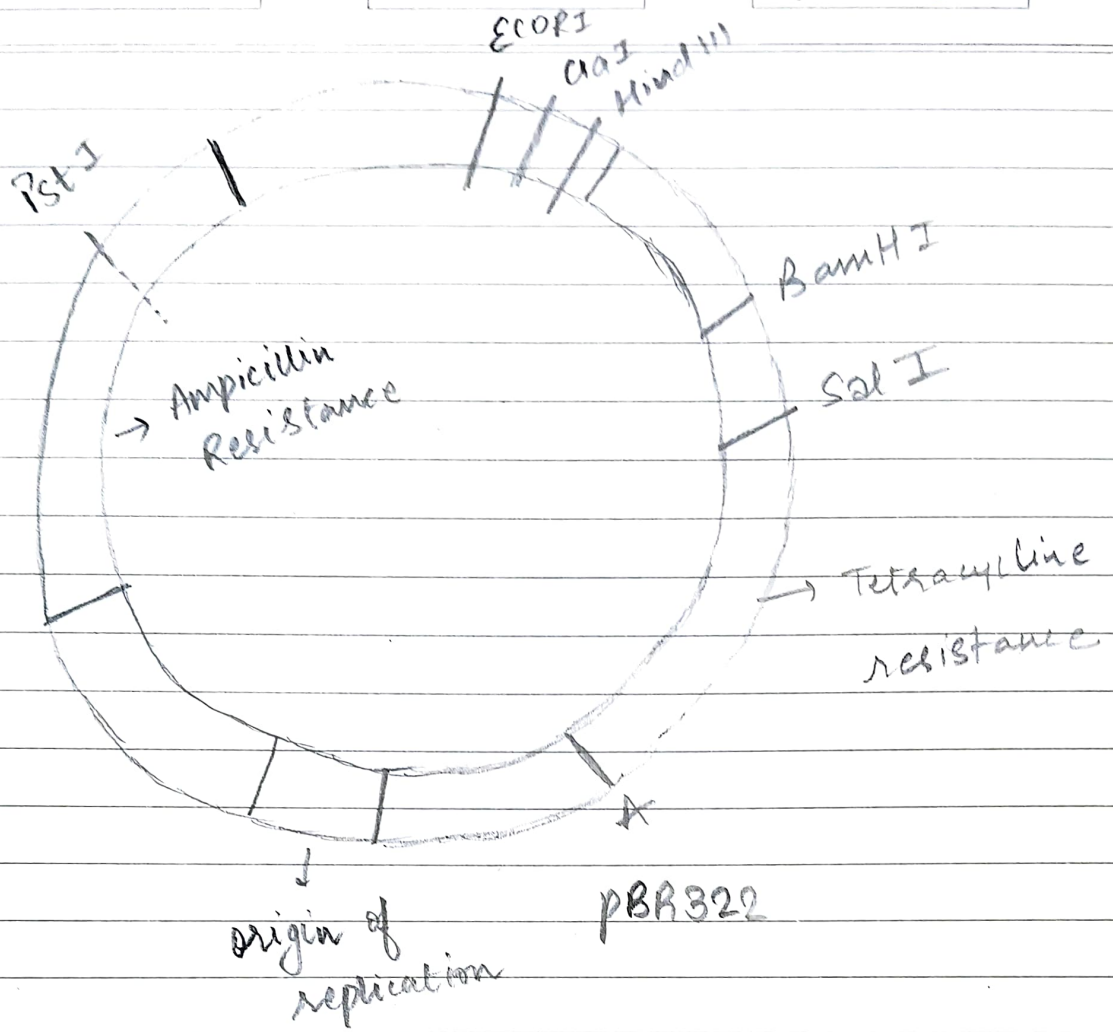
- Insertion of multiple cloning site polylinker. This is a short (~30 bp) synthetic sequence which contains unique restriction sites for a variety of common restriction nucleases (pre-existing restriction sites for these enzymes will be deleted

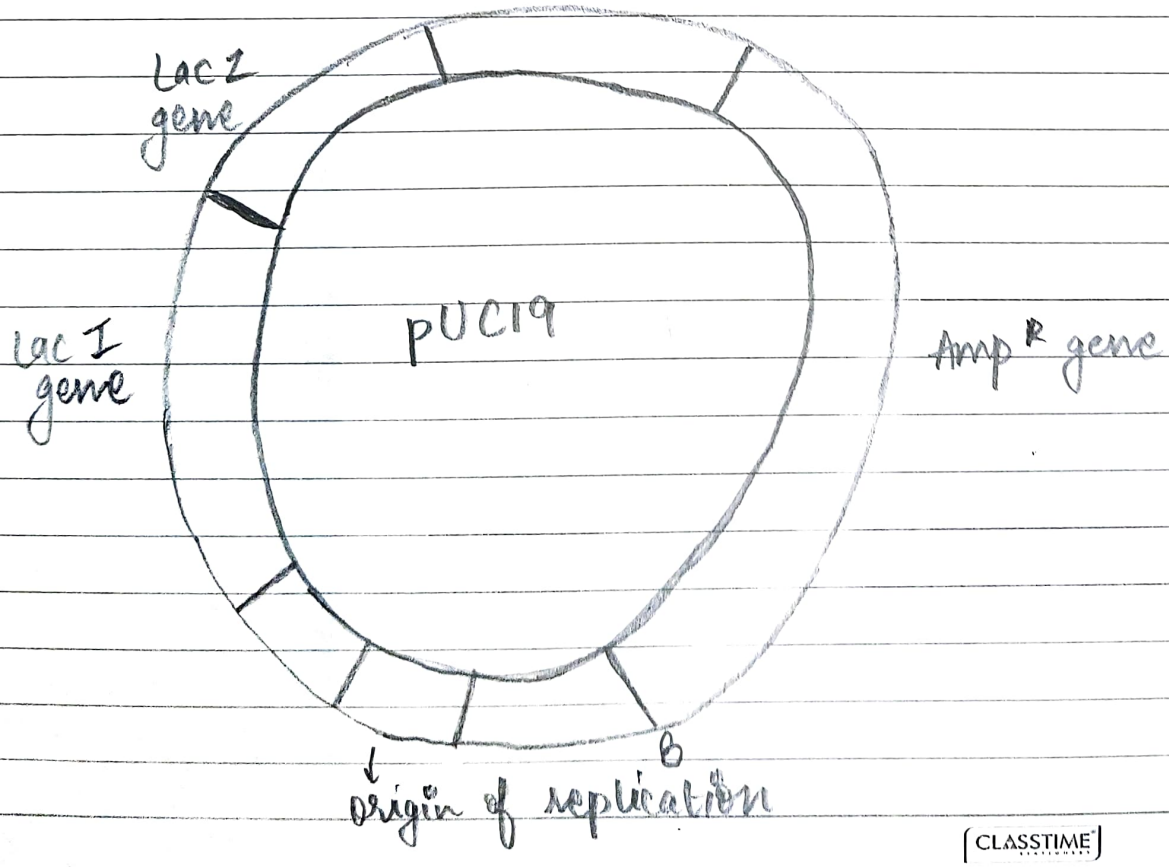
from the plasmid if necessary to ensure the presence of unique cloning sites).

- Insertion of an antibiotic resistance gene. The host cells that are used must naturally be sensitive to the antibiotic in question so that any vector molecule which transforms a host cell can confer antibiotic resistance. By plating transformed cells on a medium containing the antibiotic, only those cells that have been transformed by vector molecules survive.

- Insertion of a selection system for screening of recombinants. Typically this involves arranging for the multiple cloning site polylinker to be inserted into an expressible gene or gene fragment within the plasmid.

→ An example of a widely used cloning vector is pBR322, which replicates in E. coli. In pBR322, p stands for plasmid and BR stands for its developer Bolivar and Rodriguez. '322' distinguishes this plasmid from others developed in the same laboratory. It is small (only 4361 base pairs) and maintain in the host is relatively high copy number, 20-30 copies per cell.


EcoRI
ClaI
HindIII
PstI
BamHI
Sal I
Ampicillin Resistance
Tetracycline resistance
A
origin of replication
pBR322


lac Z gene
lac I gene
pUC19
Amp^R gene
B
origin of replication

→ The valuable features of pBR322 have been enhanced by the construction of a series of plasmids termed pUC. The plasmid vector pUC19 (2,686 bp long) contains a polylinker with unique cloning sites for multiple restriction nucleases and an ampicillin resistance gene to permit identification of transformed cells. In addition, selection for recombinants is achieved by insertional inactivation of a complement- of the β-galactosidase gene, a complementary portion of this gene being provided by using a specially modified E. coli host cell.

Bacterial Artificial Chromosome (BAC)

→ BAC cloning vectors were developed by Mel Simon & his colleagues. BAC vectors are maintained in E.coli as large single copy plasmids and contain insert of 50-300 kb. BAC vectors contain replication, F-plasmid genes that control plasmid replication and plasmid copy number and the bacterial chloramphenicol acetyl transferase gene for plasmid selection.

2. Cloning vectors based on viral DNA

Viral vectors, in which the gene or genes of interest are incorporated into the genome of a virus. Because viruses infect cells with high efficiency, the cloned genes can be introduced into cells at a significantly

higher frequency than by simple transformation. Some viral vectors are specialized for producing high levels of proteins encoded by the cloned genes and other viral vectors, such as the bacterial M13 based vectors, are designed to facilitate sequencing & the generation of mutations in cloned genes.

Cloning Vector based on λ phage

Lambda phage infects bacteria E. coli and replicates by a lytic or lysogenic pathway. Its genome is consists of single linear ds DNA of ~48Kb. However, at either end of the molecule is a short 12-nucleotides stretch, in which the DNA is single-stranded described as cohesive ends or sticky ends. 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. The second role of the cos sites is rather different, and comes into play after the prophage 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, in which a continuous DNA strand is "rolled off" the template molecule. The result is a catenate consisting of a series of linear lambda genomes joined together at the cos

sites. The role of the cos sites is now to act as recognition sequences for an endonuclease that cleaves the catenate at cos sites, producing individual lambda genomes.

In the lytic pathway, viral functions are fully expressed : viral DNA and proteins are quickly produced and packaged into viral particles, leading to the lysis of the host cell and release of virus particles, or virions.

In the lysogenic pathway, the phage DNA becomes inserted into the host-cell genome and can be replicated together with host cell DNA for many generations, remaining inactive. Certain environmental changes can trigger the expression of this dormant viral DNA, which leads to the formation of progeny virus and lysis of the host. Large segments of the 48-Kb DNA of lambda phage are not essential for productive infection and can be replaced by foreign DNA, thus making lambda phage an ideal vector. Several major types of cloning vector have been developed by modifying phage lambda.

Replacement λ Vectors.

Only DNA molecules from 37 to 52 Kb in length can be stably packaged into the λ particle. The central segment of the λ genome contains genes that are required for the

the lysogenic cycle but are not essential for lytic function. As a result, it can be removed and replaced by a foreign DNA fragment. Using this strategy, it is possible to clone foreign DNA up to 23kb in length, and such vectors are normally used for making genomic DNA libraries.

Insertion λ Vectors

Lambda vectors used for making cDNA libraries do not require a large insert capacity (most cDNAs are <5kb long). Design of insertion vectors often involves modification of the lambda genome to permit insertional cloning into the cI gene.

Cosmids

→ Cosmids are vectors that are hybrids of lambda phages and plasmids, and their DNA can replicate in the cell like that of a plasmid or be packaged like that of a phage. However, cosmids can carry DNA inserts about three times as large as those carried by lambda itself (as large as about 45kb). The key is that most of the lambda phage structure has been deleted, but the signal sequences that promote phage-head stuffing cos sites remain. This modified structure enables phage heads to be stuffed with almost all donor DNA. Cosmid DNA can be packaged into phage particles by using the in vitro system.

The development of cosmid vectors was based on the observation that a about 200 bp DNA sequence in the lambda genome called cos is required for lambda DNA packaging into phage particles during lytic infection. Cleavage at cos by the lambda terminase protein during phage packaging produces a 12 nucleotide cohesive end at termini of the linear lambda genome.

Recircularization of the lambda genome after bacterial infection is facilitated by base pairing between the complementary cohesive ends. Cosmid cloning vectors with DNA inserts of 30-45 Kb can be packaged in vitro into lambda phage particles, provided that the ligated double-strand DNA contains lambda cos sequences on either side of the insert DNA.

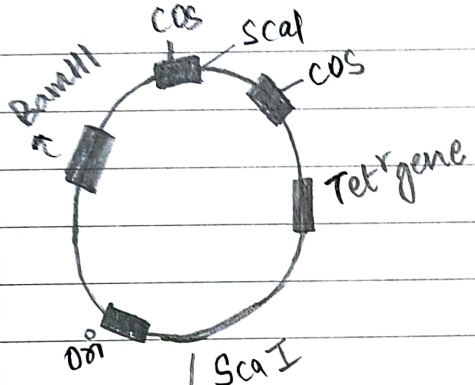
Because the lambda phage head can hold up to 45 Kb of DNA, the optimal ligation reaction in cosmid cloning produces recombinant molecules with cos sequences flanking DNA segments of about 40 Kb. E. coli cells infected with cosmid-packaged phage are able to support autonomous replication of circular plasmids because a COLE1 origin of replication and the beta lactamase gene (amp) are linked to the cos sequence

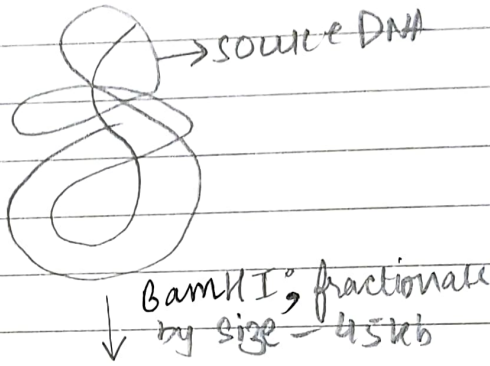
Cloning vectors based on M13 phage.

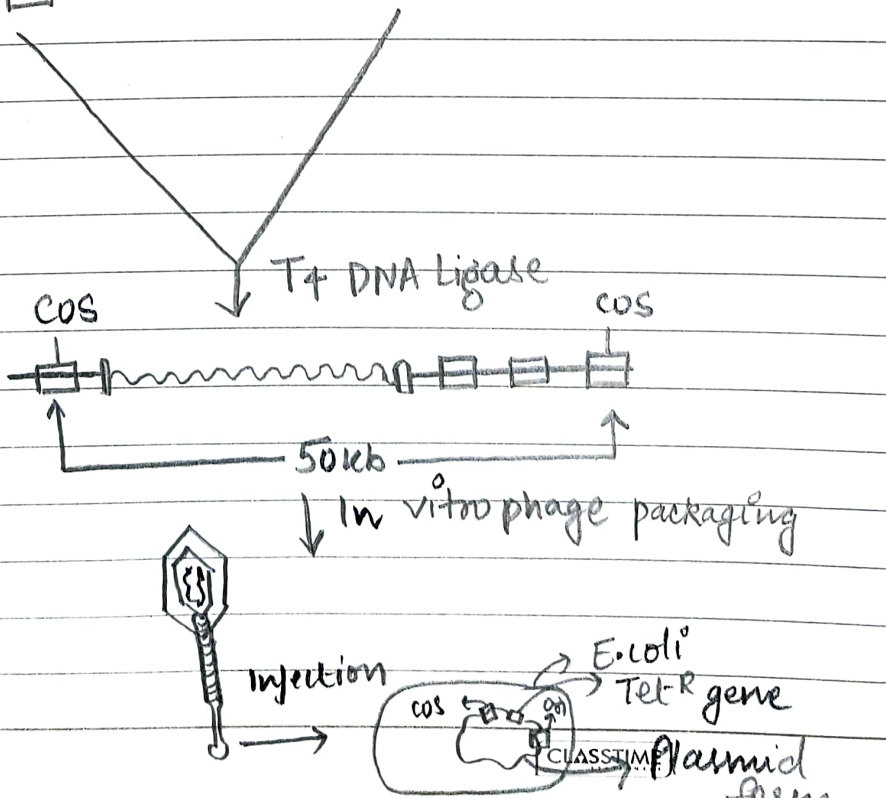
A very useful vector for cloning DNA, M13 phage is especially useful for sequencing the inserted DNA. This filamentous virus contains 6.4-kb single-stranded circle of DNA. M13 enters E. coli through the bacterial sex pilus, a protein appendage that permits the transfer of DNA between bacteria. The single-stranded DNA in the virus particle (called the (+) strand) is replicated through an intermediate circular double-stranded replicative form (RF) containing (+) and (−) strands. Only the (+) strand is packaged into new virus particles. About a thousand progeny M13 are produced per generation. A striking feature of M13 is that it does not kill its bacterial host. Consequently, large quantities of M13 can be grown and easily harvested (1 gram from 10 liters of culture fluid).

An M13 vector is prepared for cloning by cutting its circular RF DNA at a single site with a restriction enzyme. The cut is made in a polylinker region that contains a series of closely spaced recognition sites for restriction enzymes; only one of each such sites is present in the vector. A double-stranded foreign DNA fragment produced by cleavage with the same restriction enzymes is then ligated to the cut vector. The foreign DNA can be inserted in two different orientations because the ends of both

DNA molecules are the same. Hence, half the new (+) strands packaged into virus particles will contain one of the strands of the foreign DNA, & half will contain the other strand. Infection of E. coli by a single virus particle will yield a large amount of single stranded M13 DNA containing the same strand of the foreign DNA. DNA cloned into M13 can be easily sequenced.







Phagemid vectors

Phagemid vectors are plasmids DNA which contain a small segment of the genome of a filamentous phage, such as M13, fd or f1. The selected phage sequences contain all the cis-acting elements required for DNA replication and assembly into phage particles. They permit successful cloning of inserts several kilobases long (unlike M13 vectors in which such inserts tend to be unstable). Following transformation of a suitable E. coli strain with a recombinant phagemid, the bacterial cells are superinfected with a normal filamentous helper phage, such as M13, f1, which is required to provide the coat protein. Phage particles secreted from the superinfected cells will be a mixture of a helper phage and recombinant phagemids. The mixed single-stranded DNA population can be used directly for DNA sequencing of the phagemid vector adjacent to the cloning site.

Bacteriophage P1

Bacteriophage P1 vectors are very similar to λ vectors, being based on a deleted version of a natural phage genome, the capacity of the cloning vector being determined by the size of the deletion and the space within the phage particles. The P1 genome is larger than the λ genome, & the phage particle is bigger, so a P1 vector can clone larger fragments of DNA than a λ vector, up to

125 Kb using current technology.

Cloning Vectors for Eukaryotes

Cloning vectors for Yeast, S. cerevisiae

→ S. cerevisiae has a genome of approximately 2×10^7 base pairs contained in 17 linear chromosomes, and some strains possess a type of plasmid known as the 2-micron circle. This plasmid 6,318 bp and is present in a copy number of about 50 copies per cell.

Yeast Integrative plasmid (YIp)

YIp vectors lack a yeast ~~ori~~ replication origin & must be integrated into the yeast genome in order to be maintained during cell div. They are normally present at one copy per cell and are very stable. It consists of segment of the E. coli plasmid pBR322 containing a selectable antibiotic resistance gene (Ampicillin) and a bacterial replication origin which facilitate selection and amplification in E. coli.

Yeast- Replicating Plasmids (YRp)

YRp vectors contain an Autonomous Replicating Seq. (ARS) of chromosomal origin in addition to the elements found in YIps. YRps are capable of autonomous replication, are present at 20-200 copies per cell.

Yeast Centromeric Plasmids (YCp)

→ YCp vectors contain both an ARS and a yeast-centromere. The ARS can be either chromosomal or 2μ in origin. YCps are normally present at one copy per cell, can replicate without integration into a chromosome and are stably maintained during cell division.

Yeast artificial chromosomes (YAC)

M. olsen and colleagues described the first generation of yeast cloning vectors that were based on artificial chromosome. YACs is used for cloning very large DNA fragments. These vectors are propagated in S. cerevisiae rather than in E. coli and are based on chromosomes, rather than on plasmids or viruses.

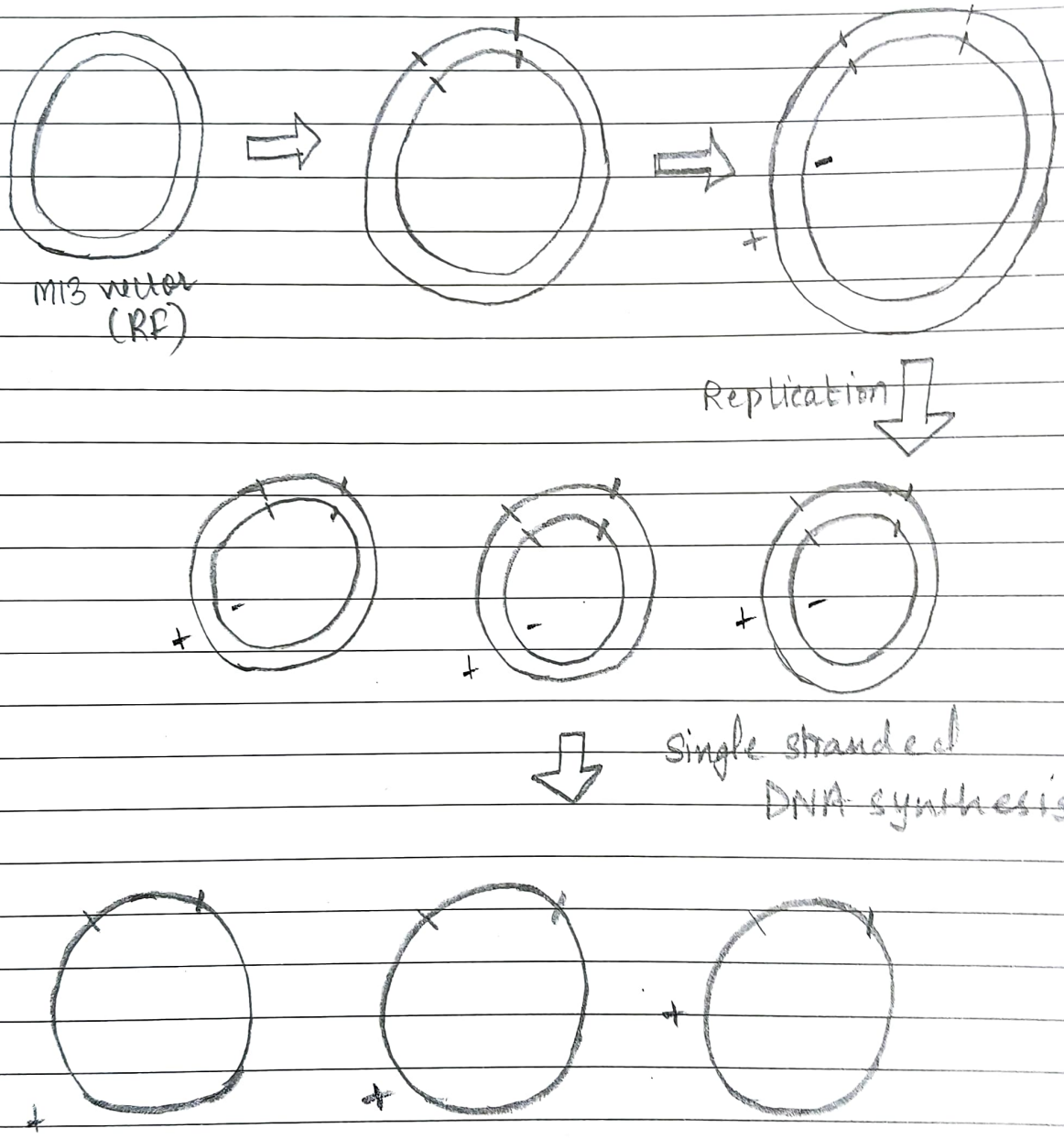
The essential functional components of chromosomes are:

Centromeres, required for disjunction of sister chromatids in mitosis and of homologous chromosomes at the first meiotic division;

Telomeres, required for complete replication of linear molecules and for protection of the ends of the chromosome from nuclease attack;

Autonomous replicating sequence (ARS) elements,

acts as origin of replication and Selectable marker to select positively for chromosomal maintenance.



Production of single stranded recombinant DNA using M13.

Shuttle vectors

→ Shuttle vectors are plasmids capable of propagating and transferring (Shuttling) genes between two different organism, one of which is typically a prokaryote (E. coli) and others a eukaryote (e.g., yeast). Shuttle vectors must have unique origins of replication for each cell type as well as different markers for selection of transformed host cells harboring the vector.

Vector	Host	Insert Size
M13	E. coli	1-4 kb
Plasmid	E. coli	1-5 kb
λ phage	E. coli	5-25 kb
Cosmids	E coli	35-45 kb
P1 phage	E. coli	70-100 kb
PACs	E. coli	100-300 kb
BACs	E. coli	≤ 300 kb
YACs	S. cerevisiae	200 - 2000 kb

Cloning vectors for plants

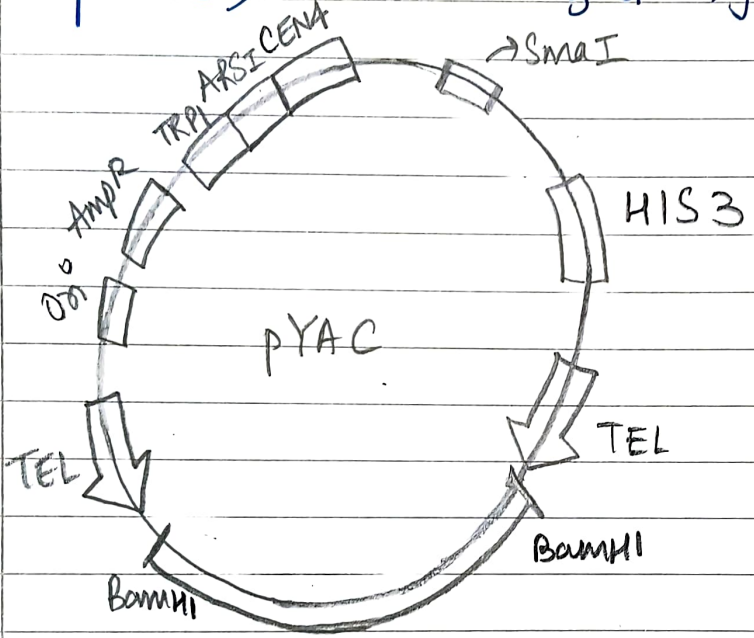
Cloning vectors for plants are either plasmid or viral genome based.

- Plasmid based vector
- Viral genome based vector

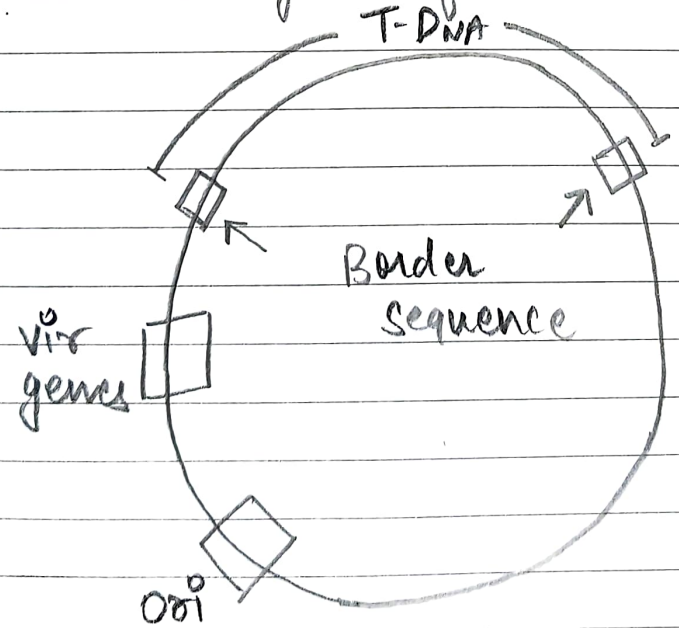
Ti plasmid based vector

Ti plasmid present in Agrobacterium tumefaciens. A. tumefaciens is naturally occurring, gram -ve, soil bacteria with two common species, viz. A. tumefaciens and A. rhizogenes. These are being considered as natural genetic engineers for their ability to transform plants. A. tumefaciens cause crown gall disease (when A. rhizogenes infects plants, the production of adventitious roots rather than tumour, is induced at the site of infection) by transferring a defined segment of DNA (called T-DNA) from its tumor inducing (Ti) large sized plasmid of about 200 kb into the nuclear genome of cells in an infected wound on many dicotyledonous plants. T-DNA varies in size b/w 15 and 30 kb depending on the strain. T-DNA carry genes for phytohormone (auxin and cytokinin) & opines. The overproduction of phytohormones at the site of infection is responsible for the proliferation of wound cells into a gall (tumour) that can harbour a population of infection is responsible for the proliferation of wound cells

into a gall (tumour) that can harbour a population of infection is responsible of wound cells into a gall (tumour) that can harbour a population of infection is responsible for the bacteria. Unusual compound opines (octopine, nopaline) are catabolized by Agrobacterium.



Schematic diagram of a YAC cloning vector.



Schematic representation of a Ti plasmid.

The movement of the T-DNA is mediated by genes in another region of Ti plasmid called vir genes (virulence genes). The virulence genes are not themselves transferred with the T-DNA but mediate the transfer. The vir genes caused, T-DNA transfer to plant cell is analogous to transfer of plasmids during bacterial conjugation.

Following transfer, T-DNA get integrated with the host genome. These properties of Agrobacterium DNA transfer system are invaluable for developing a powerful vector system for plant transformation. Any genes put in the T-DNA region, gets transferred to the plant genome. The DNA is inherently stable once in the plant genome since neither the border nor the virulence genes are transferred.

The Ri plasmid of A. rhizogenes and Ti plasmids are very similar, the main difference being that transfer of the T-DNA from an Ri-plasmid to a plant result not in a crown gall but in hairy root disease.

Viral vectors

The potential of few plant viruses as cloning vectors has been explored. One major problem with vast majority of plant viruses is that they have RNA genome not DNA genome. RNA viruses are not useful as potential cloning vectors. Only two classes of DNA virus are known to infect higher

plants, the caulimoviruses (dsDNA) and geminiviruses (ssDNA), & neither is ideally suited for gene cloning.

Cloning vectors for animals

For Insects

P element, a transposon, is used as a cloning vector in Drosophila. P element are 2.9 kb in length and contain three genes flanked by short inverted repeat sequences at either end of the element. The genes code for transposase, the enzyme that carries out the transposition process. The inverted repeats form the recognition sequences that enable the enzyme to identify the two ends of the inserted transposon. The vector is a plasmid that carries two P element resulting in disruption of its transposase gene. But the second P element carried by the plasmid has an intact version of the transposase gene that provides transposase enzyme.

09/10

MS 08/04/24

Thanks.