Genetic engineering in plants

Genetic engineering or recombinant DNA (rDNA) technology involves artificial transfer of genes or gene fragments from one organism to another to produce novel traits in the recipient living organism.

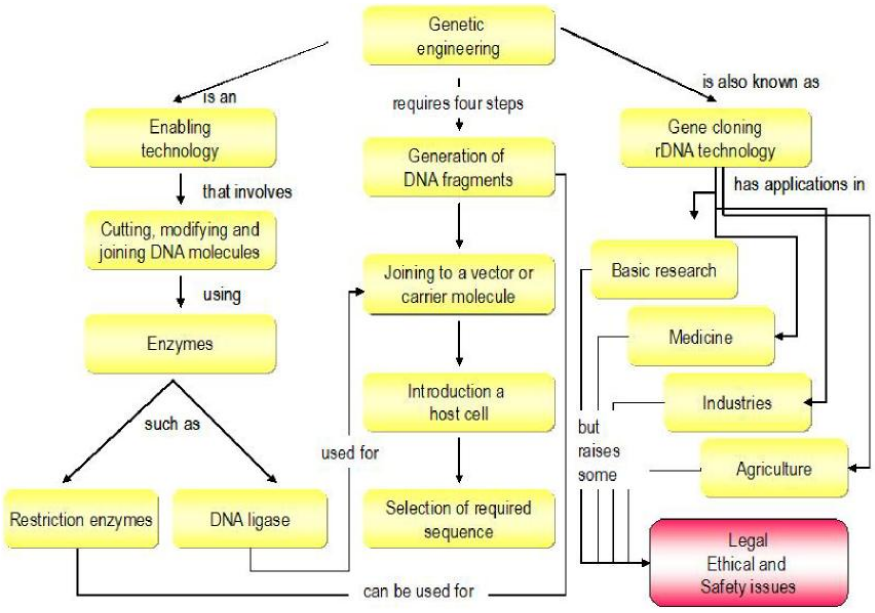
The development of **recombinant DNA technology (rDNA technology)** permitting the transfer of genetic material between widely divergent species has opened a new era of research into the structure and function of the genome. The rDNA technology is defined as "the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation. The rDNA technology has provided the means to achieve:

1) The fractionation of individual DNA components of complex genomes
2) The amplification of cloned genes
3) The opportunity to study the expression of individual genes thus cloned and
4) The potential to create new genetic combinations

There are several other terms that can be used to describe the technology, including **gene manipulation, gene cloning, genetic modification** and **genetic engineering.** The term genetic engineering is often thought to be rather emotive or trivial, yet it is probably the label that most people would recognize.

Any rDNA experiment has four essential steps:

1) Generating DNA fragments
2) Cutting and joining the DNA fragments to vector DNA molecules
3) Introducing the vectors carrying the foreign DNA into host cells where they can replicate and
4) Selecting the clone(s) of recipient cells that have acquired the recombinant DNA molecules



Steps involved in rDNA technology

Generating DNA fragments

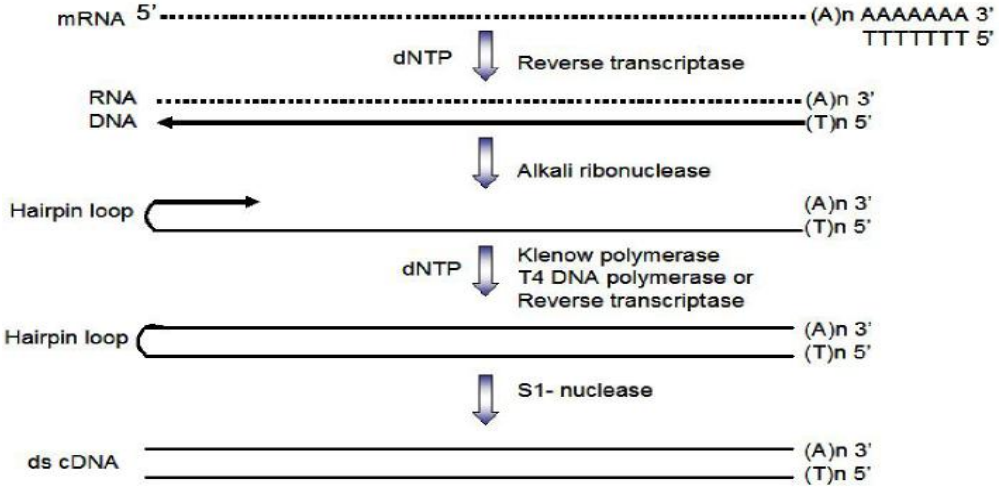
One of the most important problems prior to rDNA experiment is to separate the DNA fragments from the total genomic DNA. This is normally accomplished either by fragmentation of DNA or synthesis of new DNA molecule. The fragmentation of DNA molecule can be achieved by mechanical shearing. The long thin threads which constitute duplex DNA molecules are sufficiently rigid to be very easily broken by shear forces in solution. In this method, high molecular weight DNA is sheared to population of molecules with a mean size of about 8kb pairs by stirring at 1500 rpm for 30 minutes. Breakage occurs essentially at random with respect to DNA sequence producing termini consisting of short single stranded regions which may be repaired later. The other sophisticated technique available to generate DNA fragments involves using restriction endonucleases about which discussion is made in the subsequent section. Other two possible sources for generating DNA fragments for cloning are complementary DNA (cDNA) synthesis using mRNA as a template and artificial synthesis of DNA molecule.

cDNA synthesis

Fundamental differences exist between the genomes of prokaryotes and eukaryotes. In prokaryotes, the coding sequences (exons) are not intervened by non-coding sequences (introns) whereas in eukaryotes the genes are generally split; the coding regions are

interspersed with non coding DNA. This makes the expression of eukaryotic genes in prokaryotes a tough task.

Synthesis of cDNA from mRNA



To overcome this problem, cDNA synthesis or artificial DNA synthesis can be well exploited. In cDNA synthesis, the eukaryotic mRNA is used as a template to generate DNA. This can be achieved by making a complementary copy of the mRNA using the enzyme reverse transcriptase and whose function is to synthesize DNA upon an RNA template. At first, the enzyme was called **RNA dependent DNA polymerase.**

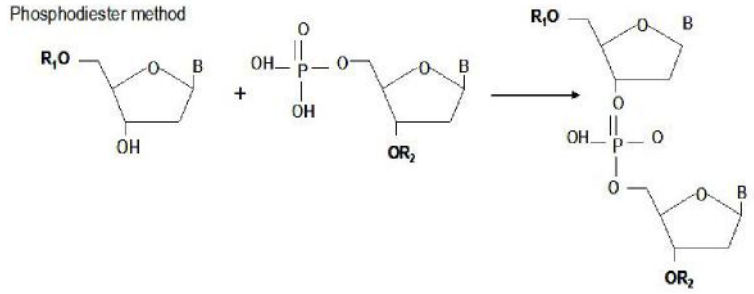
A DNA copy is made by hybridizing oligo-T primers, 10 to 20 nucleotides in length, to the 3' end of purified mRNA. Avian myeloblastosis virus (AMV) reverse transcriptase is used to synthesis a cDNA copy of the primed molecule. In the resultant RNA-DNA hybrid, the RNA can be destroyed by alkaline hydrolysis to which DNA is resistant. Thus, a single stranded cDNA is obtained which can be converted into a double stranded form in second DNA polymerase reaction. In the 3' end of the cDNA self-complementary occurs thus producing a hair-pin or snap-back structure. This acts as a primer for duplex DNA synthesis by DNA polymerase. The hair-pin loop is trimmed away by treatment with single strand specific nuclease SI, giving rise to a fully duplex molecule. The power of this technique is that only a fraction of the genome (that fraction which is transcribed into mRNA) is copied. The resulting cDNA clones can be subsequently be used as probes to identify genomic fragments contained in a genomic library.

Chemical synthesis of DNA

Although the methods for generating DNA fragments mentioned above are those most commonly used, the chemical synthesis is considered as an increasingly important method for generating DNA molecules. The chemical synthesis of specific gene sequences, regulatory sequences, oligonucleotide probes, primers and linkers is a technique in which solid phase synthesis is adopted. In chemical synthesis of DNA, two important strategies adopted are described below.

Phosphodiester method

In the phosphodiester method, 3' and 5' hydroxyl groups of deoxyribose are protected (R1 and R2). In this method, the phosphorus group between the two nucleosides is unprotected. These compounds are therefore soluble in organic solvents to a limited extent. The first significant successes, such as the synthesis of the genes for alanine and tyrosine suppressor tRNA for yeast and *E. coli* respectively were gained with the phosphodiester method.



Phosphotriester method

The phosphotriester method for the synthesis of oilgodeoxyribonucleotides proceeds essentially in two steps: 1) preparation of suitably protected monomers and 2) coupling of the monomers in the desired sequence by an appropriate phosphorylation procedure.

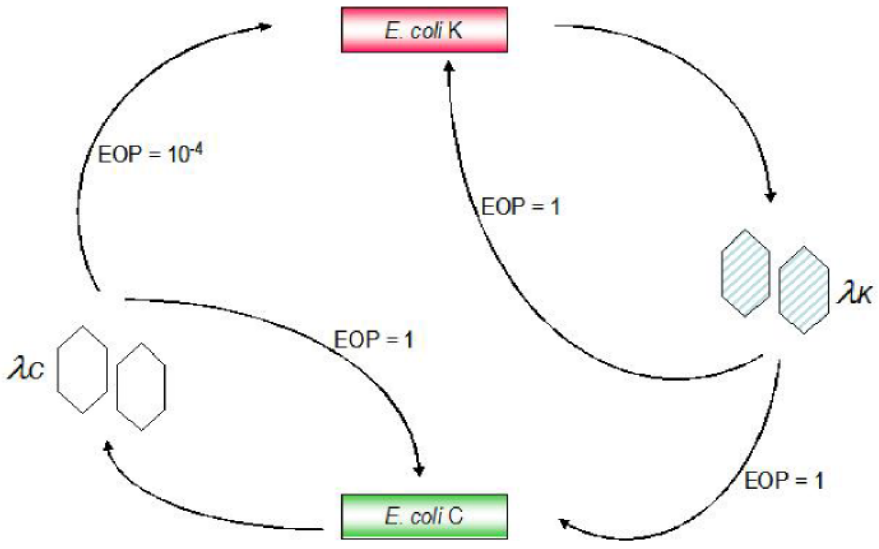
In both protocols the 3' and 5' hydroxyl groups of the deoxyribose sugar are suitably protected (R1 and R2). In the phosphotriester method a third protecting group (R3) is used for the hydroxyl group at the inter nucleotide bond. Chemical synthesis of DNA has found an extraordinary number of applications in gene technology which include synthesis of partial or total gene sequences, primers for DNA and RNA sequencing, hybridization probes for the screening of RNA, DNA and cDNA or genomic libraries and adapters and linkers for gene cloning

Cutting and joining the DNA fragments to vector DNA molecules

Restriction endonucleases: Tool for cutting DNA molecules

Techniques for cutting of DNA molecules into discrete fragments by specific enzymes were virtually unknown until the late sixties. A solution to this fundamental problem eventually grew from long standing research into the phenomenon of **host controlled restriction and modification system.**

Host controlled restriction and modification phenomenon can be well explained with the following example. If a stock preparation of phage is allowed to grow upon *E. coli* strain C and this stock is then tried upon *E. coli* C and *E. coli* K, the titres observed on these two strains will differ by several orders of magnitude, the titre on *E. coli* K being the lowest. The phages are said to be restricted by the second host strain *(E.coli* K) and the phenomenon is called **restriction.** When those phage that do result from the infection of *E. coli* K are now replated on *E. coli* K they are no longer restricted; but if they are first cycled through *E. coli* C they are once again restricted when plated upon *E. coli* K. The non-heritable change conferred upon the phage by the second host strain *(E. coli* K) that allows it to be replicated on that strain without further restriction is called **modification.** These processes can occur whenever DNA is transferred from one bacterial strain to another. Conjugation, transduction, transformation and transfection are all subject to the constraint of host controlled restriction and this process is made possible by the enzymes called **restriction endonucleases.**



Ligases: Tool for joining DNA fragments

Joining DNA fragments of various types is yet another fundamental step in rDNA technology. This process is otherwise called as ligation and is achieved by the catalytic reaction of enzymes called ligases. These enzymes catalyses the formation of phosphodiester bonds between DNA molecules. The ligase enzymes of *E. coli* and phage T4 have the ability to seal the single stranded nicks between nucleotides in a duplex DNA.

Ligation

Although the reactions catalyzed by the enzymes *of E. coli* and T4 infected *E. coli* are similar, they differ in their cofactor requirements. The T4 enzyme requires ATP, while the *E. coli* enzymes require NAD^+. In each case the cofactor is split to form an **enzyme-AMP complex.** The complex binds to the nick, which must expose a 5'- phosphate and 3'-OH group, and makes a covalent bond in the phosphodiester chain.

The other enzyme having utility in ligation is terminal deoxynucleotidyl- transferase. This adds an entire nucleotide to 3' end of the chain. It requires a source of energized nucleotides and simply adds them to the growing chain. This means that, if some DNA is mixed with terminal transferase and just one nucleotide, say the adenine nucleotide, the chain will grow as succession of adenines at the 3' end of the strand. If another chain is incubated with terminal tranferase and thymine nucleotides it will have a protruding strand that is all thymine. If the above two strands are mixed together the complementary base pairing between the protruding strands will give duplex DNA.

Prevention of self-ligation

In rDNA technology, prevention of self-ligation in vector DNA molecules or passenger DNA molecules is considered more important. Generally vector DNA molecules are highly susceptible to self ligation thus forming recircularised DNA molecules. The presence of self ligated molecules reduces the probability of recovering desired recombinant clones. Self ligation can be reduced to some extent by adopting homo polymer tailing. Wherever homopolymer tailing is undesirable other strategies like directional cloning and dephosphorylation of termini can be followed.

Directional cloning: Directional cloning is otherwise called forced cloning. This is possible in a vector having two or more target sites in a non essential portion of the

DNA. Cleavage at these sites cause the removal of non essential DNA and produce a vector molecule with two different termini which are not complementary so that the individual vectors cannot recircularise.

Dephosphorylation of termini: The main function of DNA ligase is to produce a phosphodiester bond between adjacent nucleotides if one contains a 5' PO_4 group and the other a 3' –OH group. Thus, removal of terminal 5' PO_4 groups from the cleaved DNA will prevent self ligation. Dephosphorylation of termini can be carried out by treating linearised DNA with bacterial alkaline phosphatase. The dephosphorylated DNA molecules can be religated with phosphorylated passenger DNA to produce functional recombinant DNA molecules.

rDNA techniques for the production of transgenic

Genetic engineering or recombinant DNA (rDNA) technology involves artificial transfer of genes or gene fragments from one organism to another to produce novel traits in the recipient living organism. The important tools used in rDNA technology include:

Enzymes for DNA manipulation

The first step in the construction of a recombinant DNA molecule, involves cleaving DNA molecules at specific points and recombining them together again in a controlled manner. The two main types of enzymes commonly used for this purpose are restriction endonucleases and DNA ligases. These enzymes form the backbone of rDNA technology. Restriction endonucleases cut DNA into defined fragments by targeting junction of specific sequences of the genetic coding and DNA ligases recombine them by consolidating loose bonds for creating large fragments. These enzymes are very specific in their action.

Vectors

The function of the vector is to enable the foreign genes to get introduced into and become established within the host cell. Naturally occurring DNA molecules that satisfy the basic requirements for a vector are plasmids and the genomes of bacteriophages and eukaryotic viruses. They are further classified as cloning and expression vectors depending on the stage of genetic engineering at which these vectors are used.

Expression hosts

The functional cell into which the composite DNA molecule carrying the required gene needs to be introduced is called the expression host. The choice of the best host-vector system for the expression and large-scale production of a particular protein is based on considerations of the complexity of the protein to be expressed and the yield and quantities needed.

Marker genes

Marker genes and reporter genes are utilized for selection and identification of the clones. These use phenotypic markers, identification from a gene library and DNA sequencing. DNA sequencing helps in determining the precise order of nucleotides in a piece of DNA.

Construction and Identification of recombinant DNA molecules

Recombinant DNA (rDNA) has various definitions, ranging from very simple to strangely complex. The following are three examples of how recombinant DNA is defined:

1. A DNA molecule containing DNA originating from two or more sources.
2. DNA that has been artificially created. It is DNA from two or more sources that is incorporated into a single recombinant molecule.
3. According to the NIH guidelines, recombinant DNA are molecules constructed outside of living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell, or molecules that result from their replication.

Description of rDNA

Recombinant DNA, also known as *in vitro* recombination, is a technique involved in creating and purifying desired genes. Molecular cloning (i.e. gene cloning) involves creating recombinant DNA and introducing it into a host cell to be replicated. One of the basic strategies of molecular cloning is to move desired genes from a large, complex genome to a small, simple one. The process of *in vitro* recombination makes it possible to cut different strands of DNA, *in vitro* (outside the cell), with a restriction enzyme and join the DNA molecules together via complementary base pairing.

Techniques

Some of the molecular biology techniques utilized during recombinant DNA include:

1. **The study and/or modification of gene expression patterns**

   Gene expression is the process by which a gene's coded information is converted into the structures present and operating in the cell. Expressed genes include those that are transcribed into mRNA (messenger RNA) and then translated into protein, and those that are transcribed into tRNA (transfer RNA) and rRNA (ribosomal RNA). Gene expression can be studied using microarray analysis, which is a method of visualizing the patterns of gene expression of thousands of genes using fluorescence or radioactive hybridization.
2. **Gene cloning:** Gene cloning utilizing recombinant DNA technology is the process of manipulating DNA to produce multiple copies of a single gene or segment of DNA.
3. **DNA sequencing**

   DNA sequencing is a lab technique used to determine the sequence of nucleotide bases in a molecule of DNA.
4. **Creation of transgenic plants and animals**

   A transgenic plant or animal is one who has been genetically engineered, and usually contains genetic material from at least one unrelated organism, such as from a virus, other plant, or other animal.

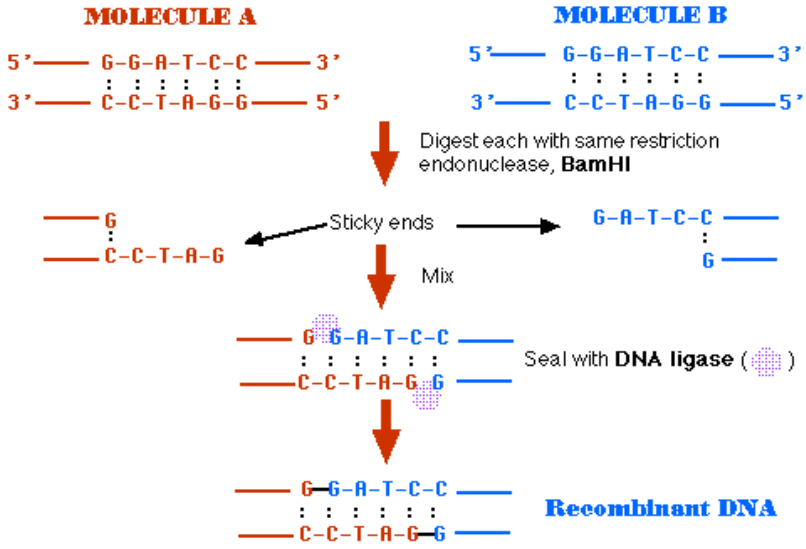
Processes

The following is a summary of the process of making recombinant DNA:

1. Treat the DNA taken from both sources with the same restriction endonuclease.
2. The restriction enzyme cuts both molecules at the same site.
3. The ends of the cut have an overhanging piece of single-stranded DNA called “sticky ends.”
4. These sticky ends are able to base pair with any DNA molecule that contains the complementary sticky end.
5. Complementary sticky ends can pair with each other when mixed.
6. DNA ligase is used to covalently link the two strands into a molecule of recombinant DNA.
7. In order to be useful, the recombinant DNA needs to be replicated many times (i.e. cloned). Cloning can be done *in vitro*, via the Polymerase Chain

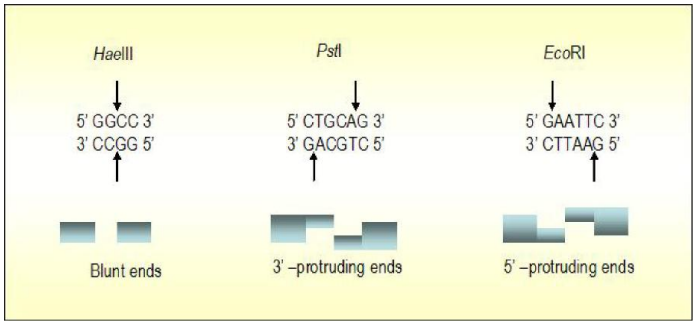
Reaction (PCR), or *in vivo* (inside the cell) using unicellular prokaryotes (e.g. E. coli), unicellular eukaryotes (e.g. yeast), or mammalian tissue culture cells.

Figure . A pictorial representation of the recombinant DNA process.



Generating DNA fragments using restriction endonucleases

Restriction endonucleases are enzymes that recognize specific sequences within duplex DNA molecules and cut the DNA at or near these sites. More than 500 different restriction endonucleases have been discovered. These enzymes can be grouped into three types *viz.* Type I, II and III. For practical purposes, the Type I and III restriction enzymes are not much used in rDNA technology. The real precision scissors are the Type II enzymes. Type II restriction endonucleases recognize and cut DNA within particular sequences of tetra, penta, hexa or hepta nucleotides which have an axis of rotational symmetry. In the following examples, different restriction enzymes cut the DNA at specific sequences as indicated by arrows.



Among the restriction enzymes, some enzymes cut the DNA molecules to give **blunt end** fragments otherwise termed as **flush end** DNA fragments and some others produce DNA molecules where one of the strands will have protruding 5' or 3' termini. These

fragments are called fragments with **cohesive ends** or **sticky ends.** The majority of the recognition sequences for restriction endonucleases are **palindromic,** that is the sequence is the same if read from 5' to 3' from both complementary strands.

The sites of cut made by endonucleases are called **target sites** or **cleavage sites** and the number of these sites in a DNA molecule depends on the size of the DNA, its base composition and the GC content of the recognition site. The number and size of the fragments generated by a restriction enzyme depends on the frequency of occurrence of the target site in the DNA to be cut. Assuming a DNA molecule with a 50 percent G+C content and a random distribution of four bases, a restriction enzyme recognizing a particular tetranucleotide sequence will be able to cut the DNA molecules into fragments at once in every 44 *(i.e.* 256) nucleotide pairs. If the enzyme is having the property of making cuts in hexanucleotide sequences means, the cuts will be made at every 46 *(i.e.* 4096) nucleotide pairs and an eight nucleotide recognition sequence 48 (65536) base pairs.

Restriction enzymes that have the same recognition sequences can be isolated from different bacterial species. Such enzymes are called **isoschizomers.** An example is provided by ***Mbo*I** *(Moraxella boris)* and ***Sau*3A** *(Staph yhcoccus aureus),* both of which recognize the sequence **GATC.** Furthermore, some restriction enzymes generate cohesive ends that can reanneal with identical termini produced by other enzymes. For instance, DNA cleaved with *Bam*HI (GGATCC) has compatible ends with DNA cleaved with *Bgl*II, *Mbo*I, *Sau*3A, *etc.*

The number of restriction fragments made by an enzyme would be reduced if there is a methylation of restriction sites. In some cases, the DNA recognition by an enzyme will not be altered by methylation and enzymes of this nature are said to be **enzymes with star activity** *e.g. Eco*Rl, *Barn*HI and *Sal*1.

Ligation strategies

In rDNA technology, sealing discontinuities in the sugar-phosphate chains, otherwise called as ligation, is vital step. This process is catalyzed by DNA ligase by repairing broken phophodiester bonds. During ligation, the enzyme’s activity is influenced by factors such as 1) substrate specificity, 2) temperature and 3) salt concentration

Ligation methods

Joining DNA fragments with cohesive ends by DNA ligase is a relatively efficient process, which has been extensively used to create artificial recombinants. If the termini of DNA fragments are not compatible, there are other methods to ligate the fragments.

Cohesive end ligation

The cohesive end ligation is possible when both the foreign DNA to be cloned and the vector DNA possess the same molecular ends. The compatible sticky ends have been generated by cleavage with the same enzyme on the same recognition sequences of both foreign DNA and vector DNA. Using DNA ligase, these molecules can be ligated without any problem.

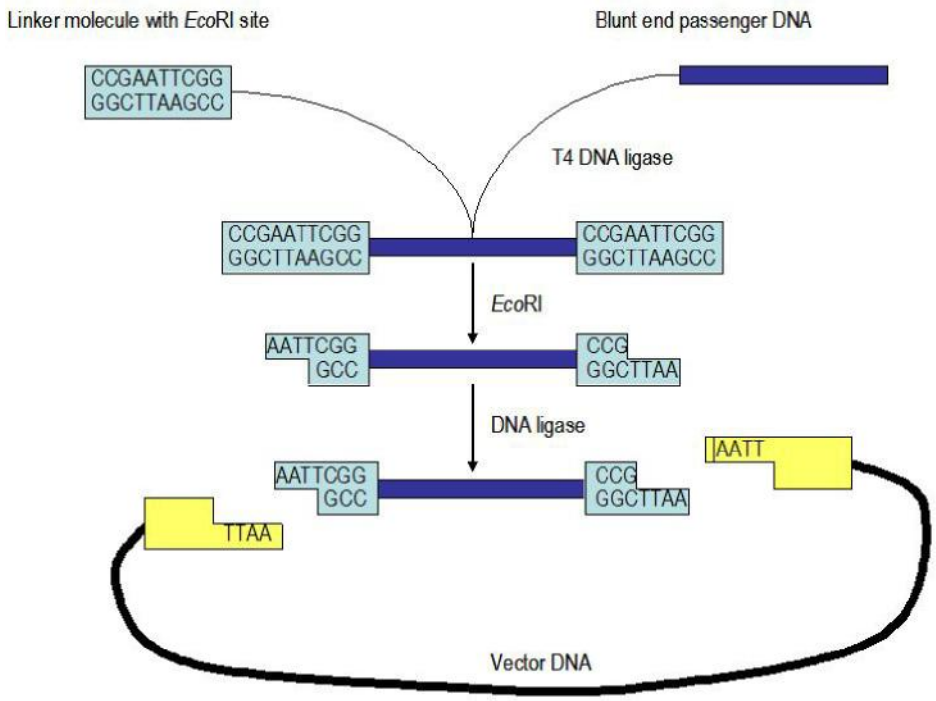
Very often it is necessary to ligate DNA fragments with different and non-compatible ends, or blunt ends with either staggered 3' or 5' ends. Incompatible DNA fragments with recessed ends can be ligated by modifying their ends by any one of the following methods *viz.,* (i) filling in recessed 3' termini and (ii) renewal of 5' protruding termini.

Blunt end ligation

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.

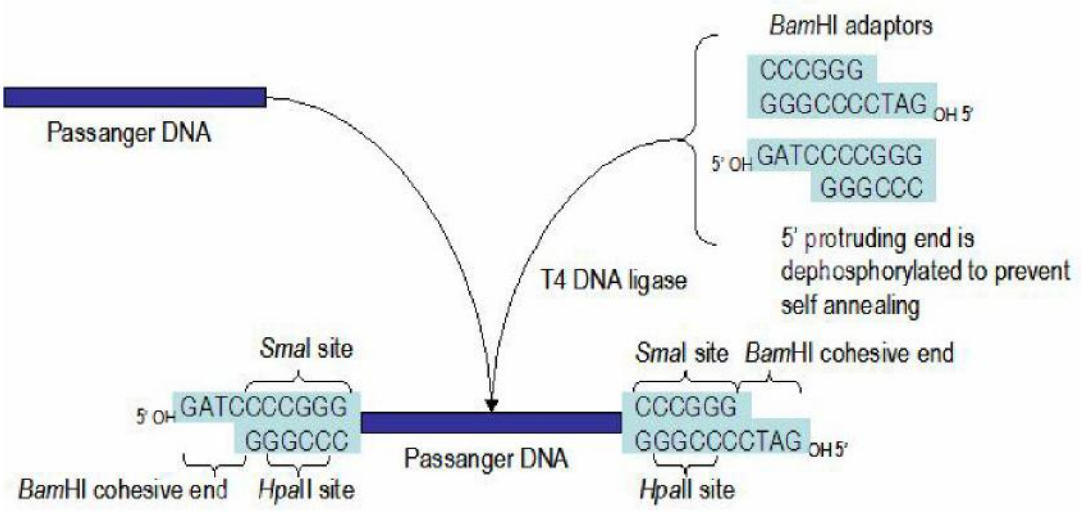
Using adaptors

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 *Bam*HI cohesive ends and sites *Hpa*II and *Sma*I can be attached to passenger DNA and

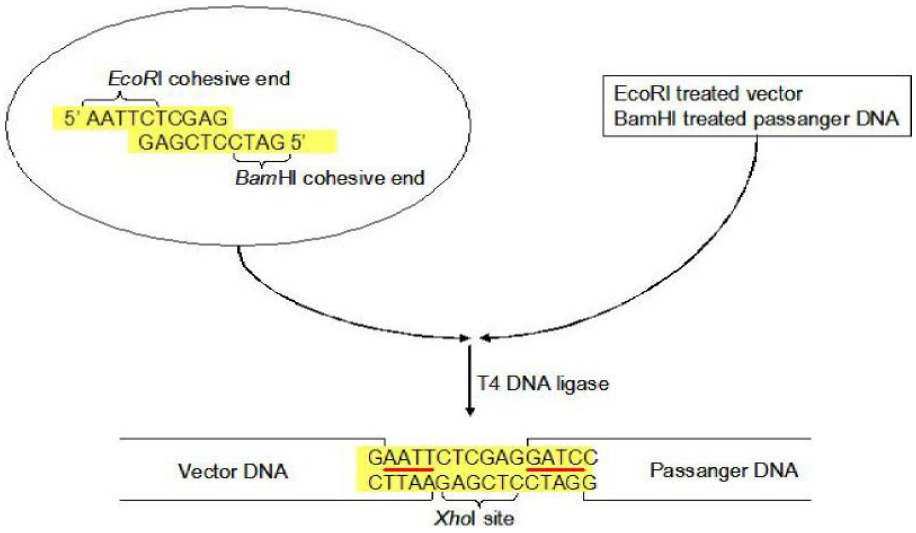
inserted into a *Bam*HI in vector. After cloning, passenger DNA can be excised from the hybrid by using any one of the enzymes that recognize the restriction sites within the adaptor region.



Use of preformed adaptors

Conversion adaptors

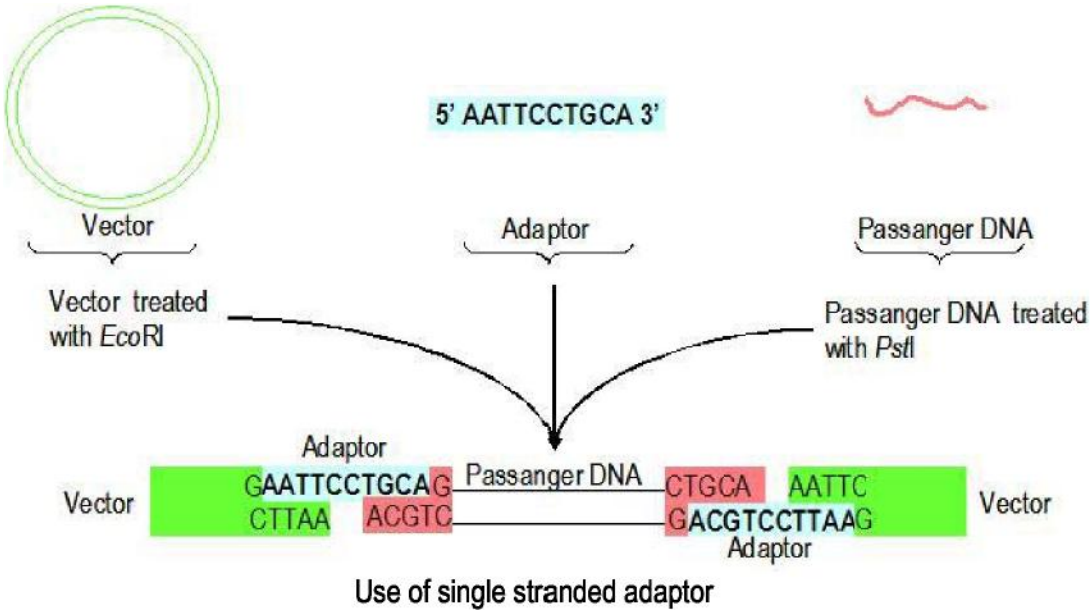
Conversion adaptors are synthetic oligonucleotides bearing different cohesive restriction termini. Such adaptors enable vector molecules that have been cleaved with one endonuclease to be joined to passenger fragments that have been cleaved with another. Often these adaptors contain internal restriction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



Use of conversion adaptor

Single stranded adaptors

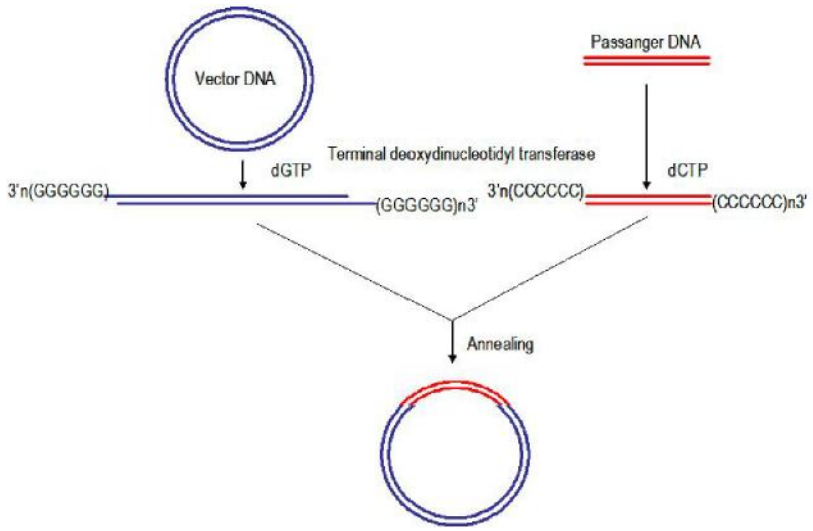
Single stranded adaptors can be used to make 3'-protruding cohesive ends compatible with 5' protruding ends. Such adaptors permit the insertion of passenger fragments into sites on vectors from which they would otherwise be precluded because of incompatible cohesive ends.



Use of single stranded adaptor

Homopolymer tailing

Homopolymer tailing is the other method adopted to clone blunt DNA molecules, especially cDNA molecules.



Homopolymer tailing

The addition of several nucleotides of single type to the 3' blunt end of DNA molecule is catalyzed by the enzyme **terminal deoxynucleotidyl transferase.** The terminal transferase permits the addition of complementary homopolymer tails (50 to 150 dA or dT long and about 20 dG or dC long) to 3' end of plasmid vector and passenger DNA. These tails can reanneal to form open circular hybrid molecules, which can be ligated *in vitro* or more commonly *in vivo* following transformations to produce functional recombinant molecules.

Selection of recombinants

Making recombinant molecules is a game with very long odds against success. Even when the bits of DNA have been joined up and inserted into cells, only very few cells out of many tens of thousands will contain the recombinant molecule and all the technical expertise in the world is no use whatsoever unless one can find the cell that contain the recombinant DNA. Techniques for selecting the few valuable cells from the mass of useless ones are thus of paramount importance.

Directional selection

The phenotypes conferred by the cloned genes on the host are used as markers of selection. All useful vector molecules carry a selectable genetic marker or have a genetically selectable property. Plasmid vectors generally possess drug resistance or nutritional markers and in phage vectors the plaque formation itself is the selectable property.

Insertional inactivation

The technique depends upon homologous recombination between DNA cloned and the host genome. If the cloned sequence lacks both promoter and sequences encoding essential regions of the carboxyl terminus of the protein, recombination with homologous genomic sequences will cause gene disruption and produce a mutant genotype. On the other hand, if the cloned fragment contains appropriate transcriptional and translational signals, homologous recombination will result in synthesis of a functional mRNA transcript, and no mutant phenotype will be observed.

Questions

1. The rDNA technology has provided the means to achieve ……

   a). Amplification of cloned genes
   b). Fractionation of individual DNA components of complex genomes
   c). Potential to create new genetic combinations
   d). All the above

2. The other terms that can be used to describe rDNA technology ……

   a). Gene manipulation
   b). Gene cloning
   c). Genetic modification
   d). All the above

3. The essential steps in rDNA technology is ……

   a). 4
   b). 5
   c). 2
   d). 7

4. In cDNA synthesis, the eukaryotic ……. is used as a template to generate DNA.

   a). mRNA
   b). tRNA
   c). rRNA
   d). None of the above

5. The enzyme reverse transcriptase is also called as ……

   a). RNA dependent DNA polymerase
   b). DNA dependent DNA polymerase
   c). RNA dependent RNA polymerase
   d). None of the above

6. The chemical synthesis of DNA is by ……

   a). Phosphodiester method
   b). Phosphotriester method
   c). Both a and b
   d). None of the above

.

7. The tool for cutting DNA molecules is/are ……

   a). Restriction endonucleases
   b). Ligases
   c). Both a and b
   d). None of the above

8. The tool for joining DNA molecules is/are ……

   a). Restriction endonucleases
   b). Ligases
   c). Both a and b
   d). None of the above

9. The important tools used in rDNA technology include ……

   a). Enzymes for DNA manipulation
   b). Vectors
   c). Expression hosts
   d).All the above

10. The rDNA technology is used for ……

a). Gene cloning
b). DNA sequencing
c).Creation of transgenic plants and animals
d).All the above

11. The restriction enzymes are grouped into …… types

a). 3
b). 5
c). 2
d).None of the above

12. The restriction enzymes used abundantly in rDNA technology is ……..

a). Type I
b). Type II
c). Type III
d).None of the above

13. The restriction enzyme with star activity is/are ……..

a). *Eco*RI
b). *Bam*HI
c). *Sal*1
d).All the above