EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 7
243-249
Year: 2016

ENZYMES INVOLVED IN RECOMBINANT DNA TECHNOLOGY

Dr. Priti Gupta*

India.

***Author for Correspondence: Dr. Priti Gupta**
India.

Article Received on 08/05/2016 **Article Revised on 28/05/2016** **Article Accepted on 19/06/2016**

ABSTRACT
Recombinant DNA means combining DNA segments from two different sources. In natural system i.e. in vivo conditions, recombination can occur during sexual reproduction in the prophase I of meiosis. But in artificial system i.e. in vitro conditions, recombinant DNA can be produced by using scientific techniques. This synthetically generated DNA can have combinations ranging from human and bacteria for insulin production, yeast and surface antigen of hepatitis virus for hepatitis B vaccine to two plants of different families forming a new transgenic plant. The first recombinant DNA was created by Stanley Cohen, H. Boyer et al in 1973. They have inserted DNA from African clawed frog into bacterium. This article deals with the basic technique of producing recombinant DNA, tools and enzymes used in generating recombinants.

KEYWORDS: RDT, Tools of RDT, Restriction enzymes, typeI, typeII, typeIIs, type III, DNA ligase, Alkaline phosphatase.

INTRODUCTION

Creating recombinants encompasses five generic steps. These steps can be modified according to the requirements of the researchers and the laboratory supporting the experiment. Steps involved in RDT are as follows.

1. Identification of gene of interest (known as **insert**) and appropriate choice of vector (known as **vehicle/carrier**).
2. Cutting the gene of interest and vector DNA with the **same** restriction enzyme.
3. Ligating the DNA molecules using DNA ligase.
4. Inserting the recombinant DNA into competent host for replication to take place.
5. Selection of the host containing only recombinant DNA (screening) and recovery of rDNA.

The product of RDT are engineered DNA, proteins and biosensors.

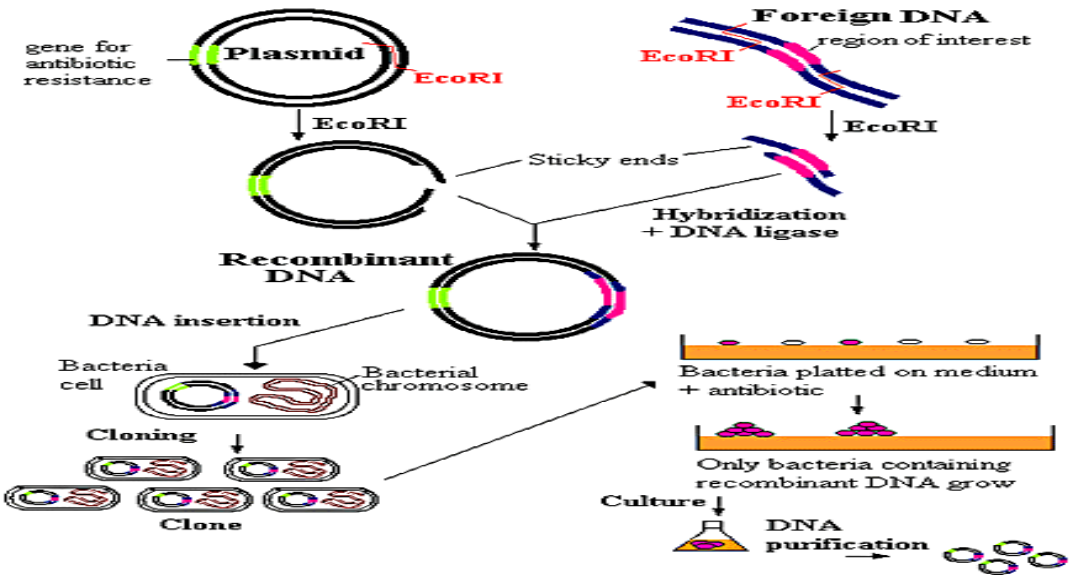


Fig1. Steps for recombinant DNA production. Source: My book on 'Fundamentals of biotechnology by Dr. Priti Gupta'.

Tools of rDNA technology
Various tools required in RDT are restriction enzymes, vectors, host and other manipulative enzymes.

Different types of Enzymes involved in Biotechnology are Nucleases (exo and endo nucleases, Restriction endonucleases, ligases, polymerases and DNA modifying enzymes such as alkaline phosphatase, polynucleotide kinase, terminal transferase and topoisomerase.

Nucleases
There are two different kinds of nucleases depending upon their site of action. These are exonucleases and endonucleases.

Exonucleases – These are the enzymes that remove nucleotides one at a time from the two ends of the DNA or RNA molecule (fig.1). The main difference between different exonucleases lies in the number of strands that are degraded when a double – stranded molecule is attacked. Some of the examples of exonucleases are as follows.

Exonuclease III – Source is *E.coli* and cuts duplex DNA from the 3'end and removes only single nucleotide. It is inactive on single stranded (ss) DNA. This enzyme creates sticky ends (5' or 3' terminus having overhangs).

Bal 31 nuclease - Source is Alteromonas spp and cuts ds DNA from both the sides. It has an additional property of acting as endonuclease on ss DNA. This enzyme creates blunt ends of DNA (i.e. none of the terminus has overhangs).

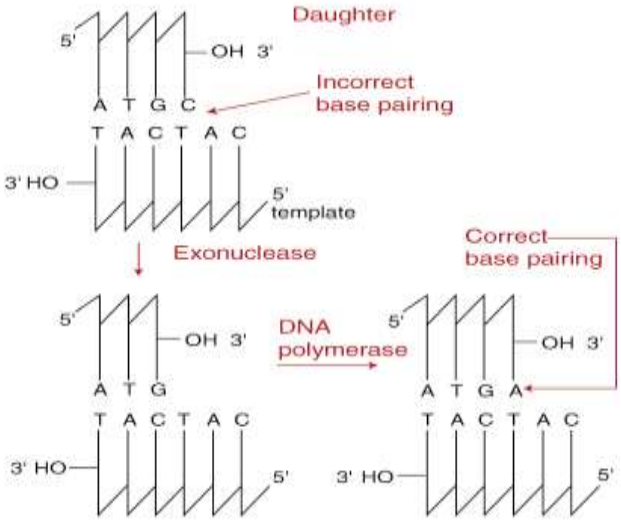


Fig1. Action of exonuclease on ds DNA.

Endonucleases – They are able to break internal phosphodiester bonds within a nucleic acid molecule (fig.2). The same criterion can be used to categorize endonucleases as used for exonucleases. Their examples include.

S1 endonuclease – Source is *Aspergillus oryzae* and digests ss DNA or RNA and high concentration of enzyme acts on ds nucleic acid. It is used to create blunt ends of ds nucleic acid. The term sticky or blunt ends are meaningless in case of single stranded nucleic acid.

DNAse I – Source is bovine pancreas. It cleaves ds or ss DNA and produces sticky ends or blunt ends depending upon the buffer used. In case of magnesium buffer, it cleaves each strand independently and randomly, hence sticky ends and with manganese buffer it cleaves at the same site producing blunt ends. It is used in elimination of DNA from RNA preparation.

RNAse – Source is same as DNAse I. It acts both as exonuclease and endonuclease and degrades RNA and hence used in the removal of RNA contamination.
Mung bean nuclease – Source is mung bean and acts on ss DNA. In high concentration, enzyme will degrade ds nucleic acid. It is used to remove ss extensions from DNA to produce blunt ends.

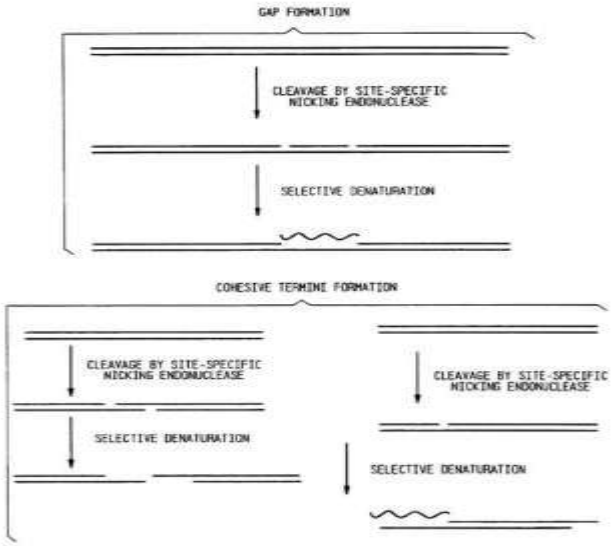


Fig.2. Action of endonuclease

Restriction endonucleases – This is most exploited enzyme category of nucleases and first discovered in early 1950s by **Arber**, **Nathans** and **Smith**. These enzymes cut the DNA molecule and the vector DNA at a specific site and make the process of recombination feasible. This enzyme system consists of two components – restriction enzymes and their corresponding cognate methylases that causes modification.

Restriction-Modification Systems
This system is found mainly in prokaryotes. A large majority of restriction enzymes have been isolated from bacteria, where they appear to serve a host-defense role**.** The principle behind is that foreign DNA, for example from an infecting virus, will be chopped up and inactivated within the bacterium by the restriction

enzyme and thus restricting the growth of foreign molecule and hence the name.

In almost all cases, a bacterium that makes a particular restriction endonuclease also synthesizes a companion DNA methyltransferase, which methylates the DNA target sequence for that restriction enzyme, thereby protecting it's own DNA from cleavage. This combination of restriction endonuclease and methylase is referred to as a restriction-modification system.

Due to their property of cutting the biomolecules i.e. DNA at a precise location, RE is also known as molecular scissors.

Nomenclature

These enzymes are named on the basis of their source of extraction according to the set rules. These rules are as follows .

- First letter of the enzyme is the initial first alphabet of the genus of the source bacterium. Eg **E** in Eco RI is the first initial of genus *Escherichia*, which is the source of this enzyme
- Subsequent two letters are the first two initials of the species of the source bacterium. Eg. **Co** in EcoRI suggests coli of *E.coli*.
- If the strain of the source is also known then the strain first letter is written afterwards. Eg **R** in EcoRI is from the strain *E.coli* strain RY13
- Roman numerals represent either of the two things 245.e. if there are many RM systems within the host then this number represents the type or this number represents the chronology of the discovery of the enzyme. Eg. **I** in the EcoRI represent that this is first discovered.

Classes of restriction enzymes

Basically, there are three types of restriction enzymes viz. type I, type II and type III but type II in turn has many other sub – types of enzyme like type A, B, S, P, T etc. of all these types type S has been found to be more famous that forced to say that there are four types of RE. table 1 below differentiate the four types of RE.

Table1: Properties of different types of restriction system.

Characteristics	Type I	Type II	Type III	Type IIs
Subunit structure	One enzyme with different subunits for recognition, cleavage and methylation	Two different enzymes that recognize the same target sequence symmetrically. The two will either cleave or modify the sequence	One enzyme with two different subunits, one for recognition and modification and one for cleavage.	Two different enzymes but recognition sequence is asymmetric
Recognition sequence	Recognize and methylates a single sequence but cleaves DNA up to 1000 bp away	Recognize short palindromic sequence (4-6 bp) and cleaves at the same site	Recognize and methylates same sequence but cleaves 24 – 26 bp away	Cleavage occurs on one side of recognition sequence up to 20 bp away
Cofactor requirement	Needs ATP and S-adenosyl methionine for cleavage	No need of ATP	Needs ATP and S-adenosyl methionine for restriction	No need of ATP

Type II Restriction Enzyme

All restriction enzymes hydrolyze the backbone of DNA between deoxyribose and phosphate groups leaving a phosphate group on the 5' ends and a hydroxyl on the 3' ends of both strands. A few enzymes will cleave single stranded DNA, although usually at very low efficiency. The restriction enzyme most commonly used in molecular biology labs is the type II as it cuts within the recognition sites and highly specific in its action generating either sticky / cohesive ends or blunt ends**.** Table 2 gives some of the examples of type II restriction enzymes and their recognition site. Restriction enzymes are **dimeric** as two dimers cut at the upper and lower side of double stranded DNA.

Table2: Restriction enzymes and their recognition sequences

Enzyme	Source	Recognition site	Average cleaved size (kb)
AluI	*Arthrobacter luteus*	AG↓CT TC↑GA	0.3
BamHI	*Bacillus amyloliquefaciens H*	G↓GATC C C CTAG↑G	7.0
EcoRI	*Escherichia coli R factor*	G↓AATT C C TTAA↑G	3.1
HaeIII	*Hemophilus aegyptus*	GG↓CC CC↑GG	0.6
HindIII	*Hemophilus influenzae Rd*	A↓AGCT T T TCGA↑A	3.1
NotI	*Norcadia otitidis-caviarum*	GC↓GGCC GC CG CCGG↑CG	< 9700
PstI	*Providencia stuartii*	C TGCA↓G G↑ACGT C	7.0
TaqI	*Thermus aquaticus*	T↓CG A A GC↑T	1.4

*Average cleaved size gives the size of the recognition sequence cleaved by enzyme

Recognition Sequence

A recognition site or **recognition sequence** is the DNA location /sequence to which restriction enzymes bind. Most of the recognition sequences are palindromes i.e. they read the same forward and backward in the 5' – 3' direction as shown in table2. R**estriction site** is the DNA sequence that is cleaved by the restriction enzyme. In case of type II enzyme both restriction site and recognition site are same (fig.3).

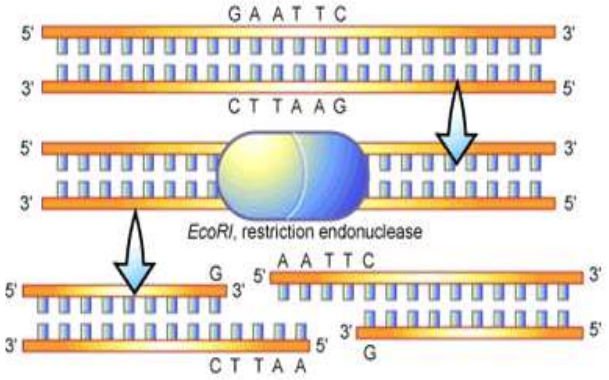
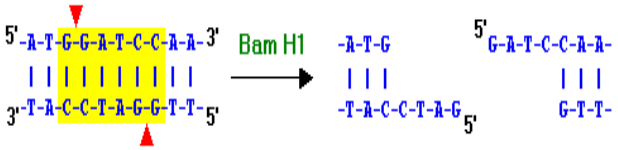


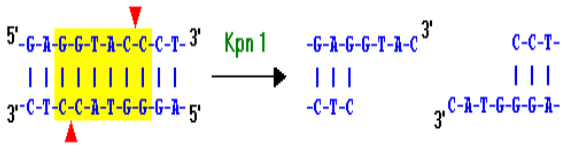
Fig.3: Recognition site (5'GAATTC3') of the enzyme EcoRI and then subsequent cleavage within the recognition site itself generating sticky ends or protruding ends or cohesive ends. These ends are easy to ligate.

The restriction enzymes most commonly used in molecular biology labs cut within their recognition sites and generate one of three different types of ends. In the diagrams below, the recognition site is boxed in yellow and the cut sites indicated by red triangles.

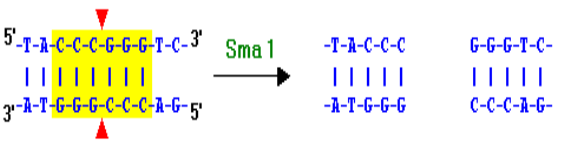
- **5' overhangs:** The enzyme cuts asymmetrically within the recognition site such that a short single-stranded segment extends from the 5' ends. BamHI cuts in this manner.



- **3' overhangs:** Again, we see asymmetrical cutting within the recognition site, but the result is a single-stranded overhang from the two 3' ends. KpnI cuts in this manner.



- **Blunts:** Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs. SmaI is an example of an enzyme that generates blunt ends.



The 5' or 3' overhangs generated by enzymes that cut asymmetrically are called ***sticky ends*** or ***cohesive ends***, because they will readily stick or anneal with their partner by base pairing.

Other common enzymes used in RDT

DNA Ligase

DNA ligases repair ss discontinuities that arise in ds DNA molecules during replication or recombination. They also join together two individual fragments of ds DNA. All living cells produce DNA ligases but the enzyme usually used in genetic engineering is that derived from *E.coli* that has been infected with T4 phage. DNA ligases close nicks in the phosphodiester backbone of DNA by forming phosphodiester bond between adjacent 5' and 3' termini. Biologically, DNA ligases are essential for the joining of Okazaki fragments during replication, and for completing short-patch DNA synthesis occurring in DNA repair process.

Mode of action – A discontinuity is simply a position where a phosphodiester bond is missing between two adjacent nucleotides that may arise in one of the strands of a ds molecule. A nick is different from the discontinuity as nick lacks one or more nucleotides. Ligases seal this discontinuity by reforming the lost phosphodiester bond at expense of one molecule of ATP at the temperature of 16 – 22^{o} C (fig.4).

- There are two classes of DNA ligases. The first uses NAD+ as a cofactor and only found in bacteria. The second uses ATP as a cofactor and found in eukaryotes, viruses and bacteriophages.
- The smallest known ATP-dependent DNA ligase is the one from the bacteriophage T_7 but is difficult to handle.
- But in RDT, the most common type of DNA ligase used is T_4 DNA Ligase because apart from catalyzing the formation of a phosphodiester bond directly between 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA with blunt or cohesive-end termini, it is ATP dependent which is the case in eukaryotes.

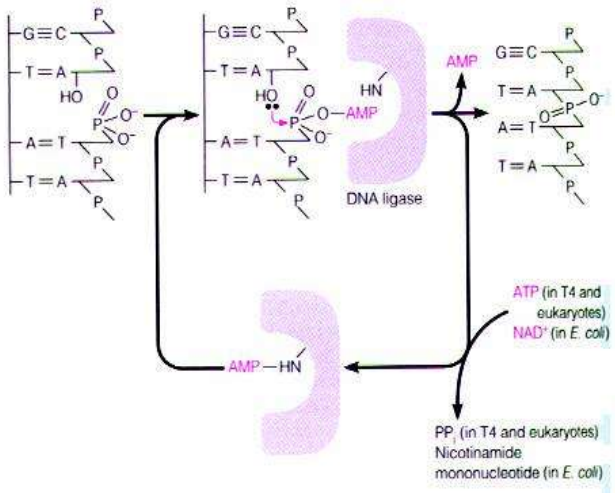


Fig.4: Active site of enzyme DNA ligase acquiring energy for the phosphodiester bond formation from the ATP leaving behind AMP. In case of eukaryotes and T4 phage energy is provided by ATP and in case of E.coli NAD^{+} is used.

Polymerases

DNA polymerases are enzymes that synthesize a new strand of DNA complementary to the existing DNA or RNA template. Therefore, polymerases require template and primer.

Three types of DNA polymerases are used routinely in biotechnology. The first one is DNA polymerase I, whose source is usually an *E.coli*. This enzyme attaches to the short single stranded region i.e. a nick in a double stranded DNA molecule and then synthesizes a completely new strand degrading the existing strand as it proceeds. Hence, DNA pol I is an example of enzyme having a dual activity of DNA polymerization and DNA degradation.

Second type of polymerase is Klenow fragment. Klenow fragment is the product of DNA pol I itself (fig.5). In fact, the polymerase and nuclease activity of the DNA pol I are controlled by different subunits of the enzyme molecule. It has 5'-3' pol activity, 3'-5' exonuclease activity and 5'-3' exonuclease activity. The nuclease activity is contained in the first 323 amino acids of the polypeptide and its removal leave the klenow fragment that retain the polymerase function but does not have cleavage function i.e. lack 5'-3'exonuclease activity. This type of polymerase finds its application in DNA sequencing.

The third type of DNA polymerase that is important in biotechnology is the **reverse transcriptase**. It is an enzyme that is involved in the replication of the several types of viruses. This enzyme has forced the scientific community to update the central dogma of life, DNA to RNA to Protein has been converted to DNA to RNA to DNA / Protein, because of its unique property of using RNA as template and synthesize DNA strand complementary to RNA. This enzyme finds its application in cDNA cloning that is used to study expressible genes.

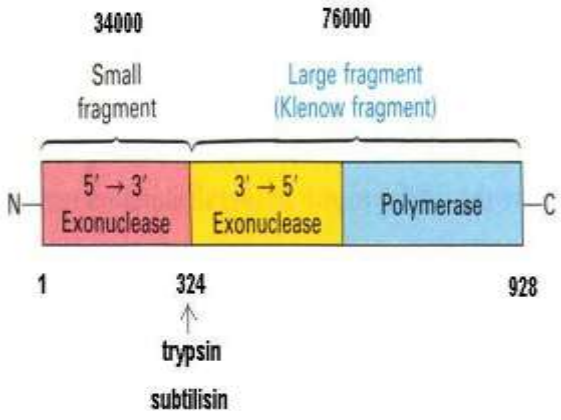


Fig.5: DNA polymerase I enzyme with all its three components and the composition of klenow fragment.

DNA modifying Enzymes

There are numerous enzymes that modify DNA molecules by addition or removal of chemical groups. The most important ones are.

Alkaline phosphatase – It removes the phosphate group present at the 5' terminus of a DNA or RNA molecule so as to inhibit the self annealing of either vector or foreign DNA molecules (fig.6). It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH – hence their name as alkaline phosphatase. Most commonly used are calf intestine or bacterial alkaline phosphatase.

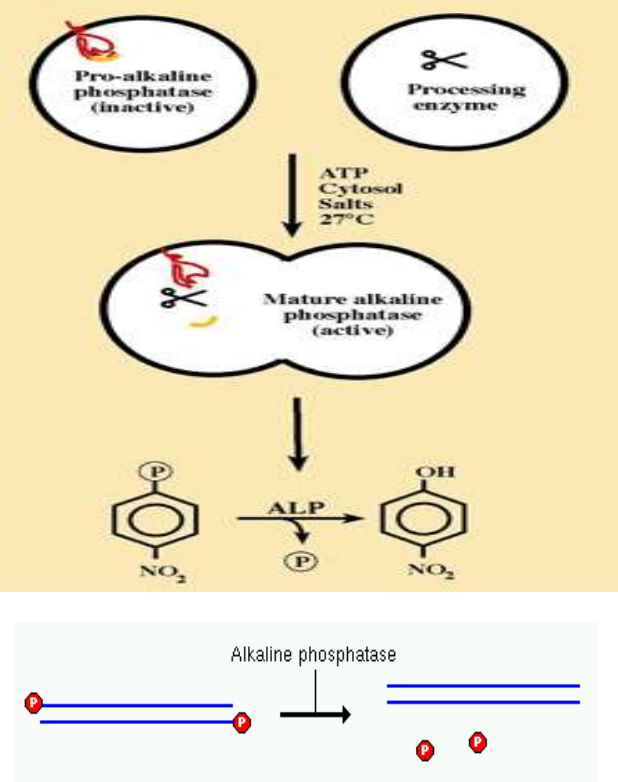


Fig.6: Action of active alkaline phosphatase. In the last step removal of phosphate group is shown.

Polynucleotide kinase – It is exactly reverse of the alkaline phosphatase as it adds phosphate groups on to the free 5' termini from the ATP molecule. It is added just before the annealing is taking place (fig.7).

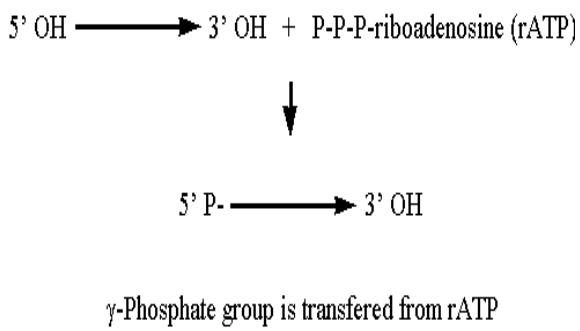


Fig.7: Reaction catalysed by the polynucleotide kinase.

Terminal deoxynucleotidyl transferases – It adds one or more deoxynucleotides on to the 3' terminus of a DNA molecule. It is a template independent polymerase. It is used to label 3'end, tailing DNA with homopolymer nucleotides (poly A tail) (fig.8).

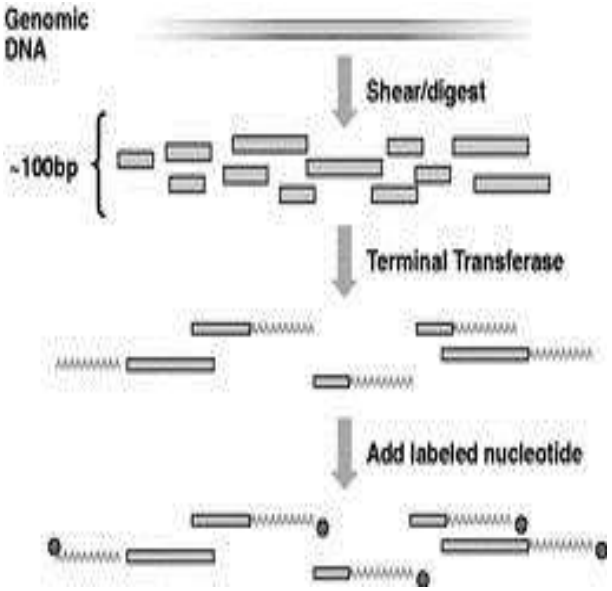


Fig.8: Terminal transferases adding the stretch of nucleotides (swirl line) to the gene fragment.

Topoisomerases.

The final class of DNA manipulative enzymes is the topoisomerases that are able to change the conformation of the covalently closed circular DNA by introducing or removing supercoils from them. Their various actions are depicted in fig.9. These molecules are most important enzymes in carrying out the replication of DNA molecule but yet to find intensive application in the biotechnology.

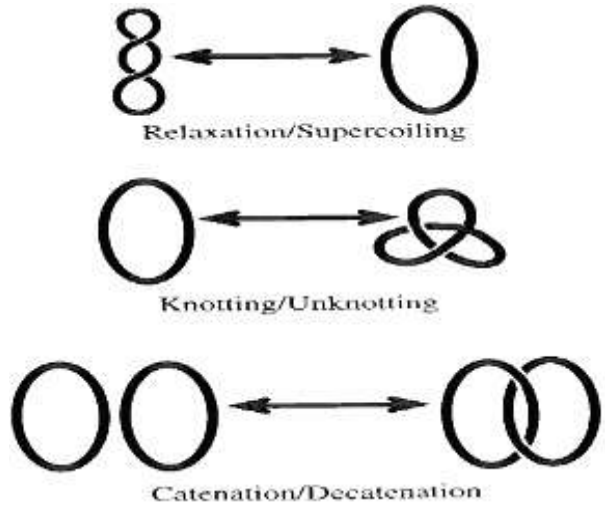


Fig. 9: Topoisomerase introduces and removes supercoils from the DNA resulting in the formation of above structures.

REFERENCES

1. Botstein, David, et al. "Construction of a genetic linkage map in man using restriction fragment length polymorphisms." *American journal of human genetics.*, 1980; 32(3): 314.
2. Fogarty, William M., and Catherine T. Kelly, eds. *Microbial enzymes and biotechnology*. Springer Science & Business Media, 2012.
3. Horton, Robert M., et al. "Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension." *Gene.*, 1989; 77(1): 61-68.
4. Kim, Yang-Gyun, Jooyeun Cha, and Srinivasan Chandrasegaran. "Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain." *Proceedings of the National Academy of Sciences.*, 1996; 93(3): 1156-1160.
5. Lahiri, Debomoy K., and John I. Nurnberger Jr. "A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies." *Nucleic acids research.*, 19914; 19(19): 5444.
6. Nelson; Cox. Lehninger principles of biochemistry. 3rd Ed. McMillan Press Ltd. London., 2003.
7. Palmer, Trevor, and Philip L. Bonner. *Enzymes: biochemistry, biotechnology, clinical chemistry*. Elsevier, 2007.
8. Primrose S; Old B; Twyman R. 2001. Principles of gene manipulation. 6th Ed. Blackwell Science, USA.
9. Telenti, Amalio, et al. "Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis." *Journal of Clinical Microbiology.*, 1993; 31(2): 175-178.
10. Vincze, Tamas, Janos Posfai, and Richard J. Roberts. "NEBcutter: a program to cleave DNA with restriction enzymes." *Nucleic acids research.*, 2003; 31(13): 3688-3691.