rDNA TECHNOLOGY

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Himalaya Publishing House
ISO 9001:2008 CERTIFIED

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Second Revised Edition: 2014

Himalaya Publishing House

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• LUCKNOW • AHMEDABAD • ERNAKULAM • BHUBANESWAR • INDORE • KOLKATA • GUWAHATI

First Edition : 2011
Second Revised Edition : 2014

Published by : Mrs. Meena Pandey for **Himalaya Publishing House Pvt. Ltd.,**
"Ramdoot", Dr. Bhalerao Marg, Girgaon, **Mumbai - 400 004.**
Phone: 022-23860170/23863863, Fax: 022-23877178
E-mail: himpub@vsnl.com; Website: www.himpub.com

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DTP by : **Pooja S.**

Printed at : M/s Sri Sai Art Printer Hyderabad. On behalf of HPH.

Preface

As we know, that current era is the time of biotechnology and especially I would say rDNA technology. In this context, there is key need to understand the concept of rDNA technology as such. Keeping in mind the basic need of students, this book is designed. All the chapters are in very simple language. The Chapter 1 deals with key DNA manipulating enzymes followed by Chapter 2 which deals with the most important part of rDNA and that is, vectors. The labeling of the nucleic acids have been described in Chapter 3 followed by Chapter 4 and 5 which overall deals with cDNA/genomic libraries, PCR and applications. Sequencing of the nucleic acid has been described in Chapter 6. Nevertheless, to gain insight into the applications of rDNA technology, Chapter 7 has been designed. The whole content is simply presented, and orderly arranged. I am fully sure that the students will gain a lot from this book and I encourage them to read and suggest modifications in the coming time. I feel satisfied if the book serves the purpose for which it is intended. I have tried to minimize typographical errors but still some might have crept in. If they are brought to my notice, I will be rectifying them in the next edition. Constructive criticism is always welcome.

Author

Preface to Second Revised Edition

A substantial amount of achievements have been made using many prokaryotic and eukaryotic organisms to understand the basic principles that govern life. Many achievements made by biotechnologists have helped in developing new branches of science such as molecular biology, rDNA technology, proteomics and genomics. Hence, biotechnology is one of most exciting and challenging field in teaching and research. Among all branches of biotechnology rDNA technology has attracted worldwide attention. It is the field that can end the world hunger in a lesser duration. Even newspapers too are giving information on all fields of biotechnology to educate people. Hence, the present book is intended to be an introduction to the subject for students.

As we know that current era is the time of biotechnology and especially, I would say rDNA technology. In this context, there is key need to understand the concept of rDNA technology as such. Keeping in mind the basic need of students, this book is designed. All the chapters are in very simple language. The Chapter 1 deals with key DNA manipulating enzymes followed by Chapter 2 which deals with the most important part of rDNA and that is, vectors. The labeling of the nucleic acids have been described in Chapter 3 followed by Chapters 4 and 5 which overall deals with cDNA/genomic libraries, PCR and applications. Sequencing of the nucleic acid has been described in Chapter 6. Nevertheless, to gain insight into the applications of rDNA technology, Chapter 7 has been designed. Chapter 8 has been designed for site directed mutagenesis. The whole content is simply presented, and orderly arranged. I am fully sure that students will gain a lot from this book and I encourage them to read and suggest modifications in the coming time. I feel satisfied if the book serves the purpose for which it is intended. I have tried to minimize typographical errors but still some might have crept in. If they are brought to my notice, I will be rectifying them in the next edition. Constructive criticism is always welcome.

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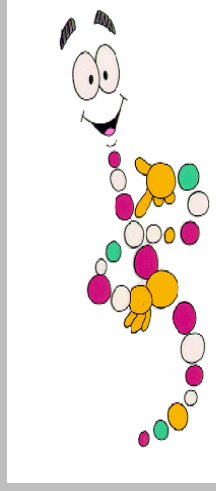


rDNA Technology: DNA Manipulating Enzymes

Until the early 1970s, DNA was the most difficult cellular molecule for the biochemist to analyze. Enormously long and chemically monotonous, the nucleotide sequence of DNA could be approached only by indirect means, such as by protein or RNA sequencing or by genetic analysis. Today, the situation has changed entirely. From being the most difficult macromolecule of the cell to analyze, DNA has become the easiest. It is now possible to excise a specific region of DNA, to produce a virtually unlimited number of copies of it, and to determine the sequence of its nucleotides at a rate of hundreds of nucleotides a day. By variations of the same techniques, an isolated gene can be altered (engineered) atwill and transferred back into cells in culture. With more difficulty, the redesigned gene can be inserted into the germ line of an animal or plant, so as to become a functional and heritable part of the organism's genome. These technical breakthroughs have had a dramatic impact on all aspects of cell biology by allowing the study of cells and their macromolecules in previously unimagined ways. They have led to the discovery of whole new classes of genes and proteins and have revealed that many proteins have been much more highly conserved in evolution than had been suspected. They have provided new means to determine the functions of proteins and of individual domains with proteins, revealing a host of unexpected relationships between them. By making available large

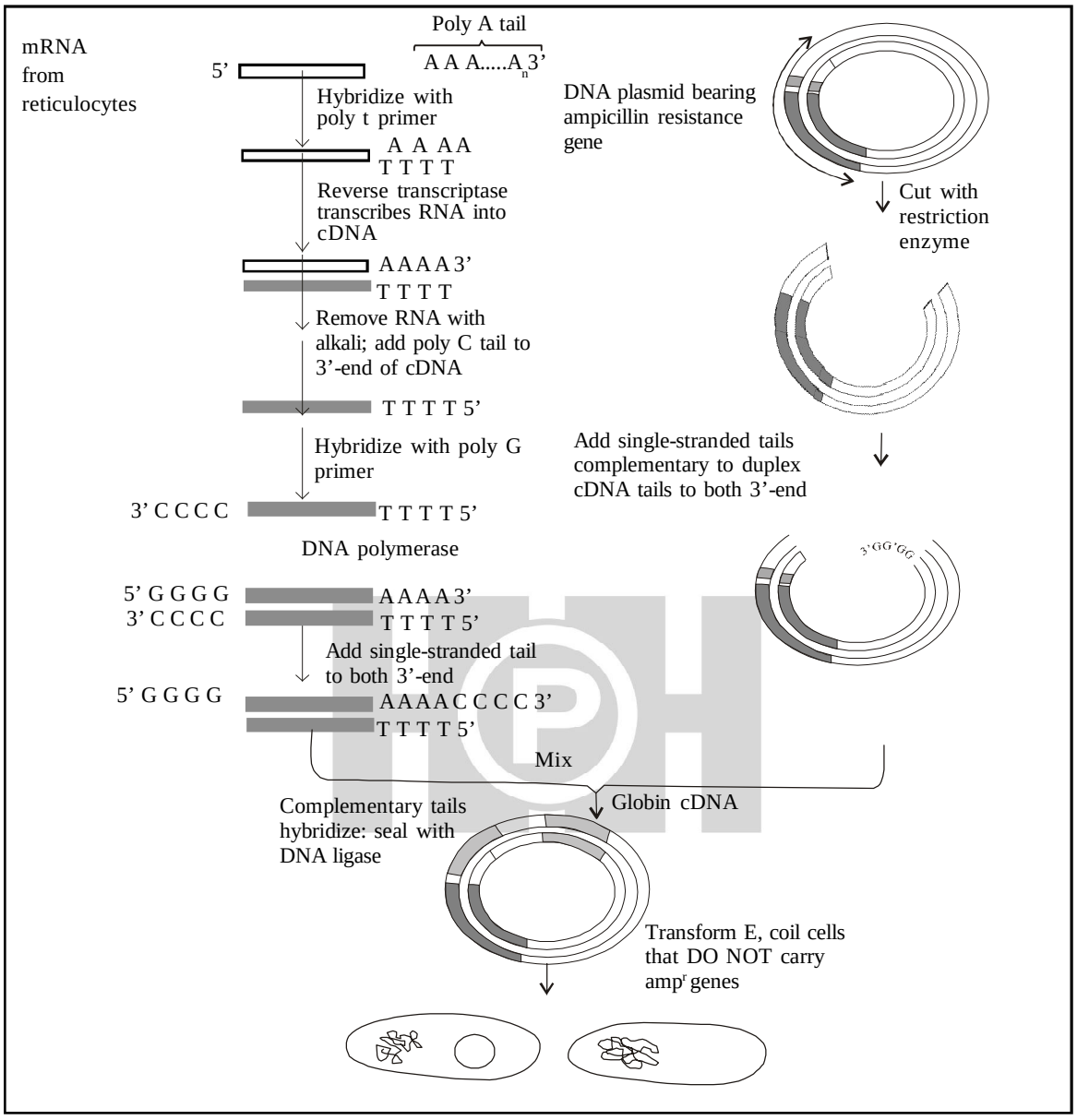


Fig. 1.1

amounts of any protein (fig.1.1), they have shown the way to efficient mass production of protein hormones and vaccines. Finally, by allowing the regulatory regions of genes to be dissected, they have provided biologists with an important tool for unraveling the complex mechanisms by which eucaryotic gene expression is regulated. Recombinant DNA technology comprises a mixture of techniques, some new and some borrowed from other fields such as microbial genetics. The most important of these techniques are the following:

1. Cleavage of DNA at specific sites by *restriction nucleases,* which greatly facilitates the "isolation" and manipulation of individual genes.
2. Rapid *sequencing* of all the nucleotides in a purified DNA fragment, which makes it possible to determine the boundaries of a gene and the amino acid sequence it encodes.
3. *Nucleic acid hybridization,* which makes it possible to find a specific sequence of DNA or RNA with great accuracy and sensitivity on the basis of its ability to bind a complementary nucleic acid sequence.
4. *DNA cloning,* whereby a single DNA molecule can be copied to generate many billions of identical molecules.
5. *DNA engineering,* by which DNA sequences are altered to make modified versions of genes, which are reinserted back into cells or organisms.

SOME MAJOR STEPS IN THE DEVELOPMENT OF RECOMBINANT DNA TECHNOLOGY

1869 **Miescher** isolated DNA for the first time.

1944 **Avery** provided evidence that DNA, rather than protein, carries the genetic information during bacterial transformation.

1953 **Watson and Crick** proposed the double-helix model for DNA structure based on x-ray results of Franklin and **Wilkins**.

1957 **Kornberg** discovered DNA polymerase, the enzyme now used to produce labeled DNA probes.

1961 **Marmur and Doty** discovered DNA renaturation, establishing the specificity and feasibility of nucleic acid hybridization reactions.

1962 **Arber** provided the first evidence for the existence of DNA restriction nucleases, leading to their later purification and use in DNA sequence characterization by Nathans and H. Smith.

1966 **Nirenberg**, **Ochoa**, and **Khorana** elucidated the genetic code.

1967 **Gellert** discovered DNA ligase, the enzyme used to join DNA fragments together.

1972-1973 DNA cloning techniques were developed by the laboratories of **Boyer**, **Cohen**, **Berg**, and their colleagues at Stanford University and the University of California at San Francisco.

1975 **Southern** developed gel-transfer hybridization for the detection of specific DNA sequences.

1975-1977 **Sanger and Barrell** and **Maxam and Gilbert** developed rapid DNA-sequencing methods.

1981-1982 **Palmiter and Brinster** produced transgenic mice; **Spradling and Rubin** produced transgenic fruit flies.

1985 **Mullis** and co-workers invented the polymerase chain reaction (PCR).

The greatest advances in molecular cell biology in the recent past have been in the analysis and manipulation of macromolecules, particularly DNA. For years, it was clear that many deep

biological secrets were locked up in the sequence of DNA. However, obtaining the sequences of long regions of DNA — not to mention altering the sequences at will — seemed a distant dream. An avalanche of technical advances has drastically changed that perspective. First came the discovery of enzymes that cut the DNA from any organism at specific short nucleotide sequences, thus generating a reproducible set of pieces. The availability of these enzymes then greatly facilitated two other important developments, DNA cloning and DNA sequencing.

Through the use of advanced enzymatic and micro logical techniques, pure pieces of any DNA can be inserted into bacteriophage DNA or other carrier DNA to produce *recombinant DNA*. The recombinant molecule can be introduced into bacteria or yeast cells, and cells bearing specific recombinant molecules can be selected. These can then be grown in unlimited quantities. This procedure is referred to as *cloning* a particular DNA sequence; the joining of sequences from two DNA molecules to form a recombinant DNA molecule is accomplished by the use of *recombinant DNA technology*.

In addition to procedures that allow the selection and production of large amounts of pure DNA, a number of other techniques have been developed. For example, *rapid DNA sequencing*, which came into being in the late 1970s, was made possible by advances in certain chemical and enzymatic techniques: fragments of DNA can be labeled with radioactive tracers, and then any fragments containing up to about 500 nucleotides could be separated by gel electrophoresis, a technique that uses an electric field to separate nucleotide chains on the basis of their length. There was no longer any obstacle to obtaining the sequence of a DNA molecule containing 10,000 or more nucleoties. Suddenly, any DNA was accessible to isolation and to sequencing. Techniques were soon developed for modifying and rearranging DNA sequences. These procedures have been coupled with others representing advances in cell biology to test the recombinant DNA molecules for changed biological activity. Finally, selected DNA fragments that encode proteins of particular interest have been transferred to bacteria and to other cells, where the transferred DNA has caused the production of these proteins. Almost overnight, these techniques of *molecular genetics* have become the dominant approach to the study of many basic biological questions particularly questions concerning the nature of genes and how they work in eukaryotic cells. The power and the success of the new technology have given birth to many hopes for the practical use of our ever increasing biological knowledge to benefit human beings.

Various steps of molecular genetics are:

1. Identification of Gene
2. Selection of a suitable vector: As per the mode of cloning, various vectors are available which can be used to clone the DNA, e.g., plasmids, cosmids, BAC, YAC etc.
3. Ligation of desired DNA: DNA can be ligated by suitable enzymes into the vector. The rDNA molecule then can be amplified. Large number of clones can be generated.

4. Transfer of Desired Gene: The rDNA molecule can be transferred to the host selected, multiplied and produced transgenic plants.
5. Expression of DNA: The inserted rDNA can be altered for the expression of new product.

Nowadays keeping in mind the potential of genetic engineering, many potential application can be done for mankind.

Important Applications of rDNA Technology

The Gene can be modified or changed as per the desire of scientist, e.g.,

1. Induce of silent gene.
2. Gene can be silenced by RNAi.
3. Gene can be overexpressed.
4. One can change the spatial and temporal expression of a gene.
5. Gene can be altered as per the environmental condition.

Hence, rDNA technology can do immense potential for the Science and Technology as shown in the Fig. 1.2 below:

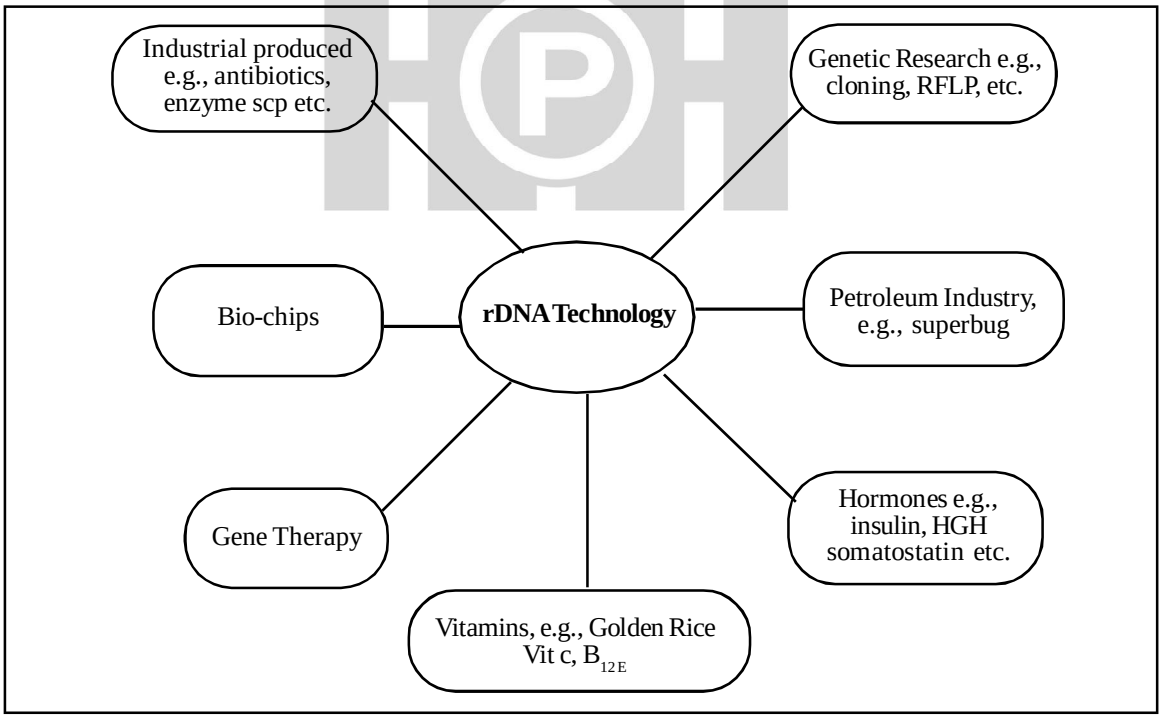


Fig. 1.2

DNA Manipulating Enzymes

Nucleases

Nucleases degrade DNA by breaking the phosphodiester bonds. There are two different kinds of nucleases. (Fig. 1.3).

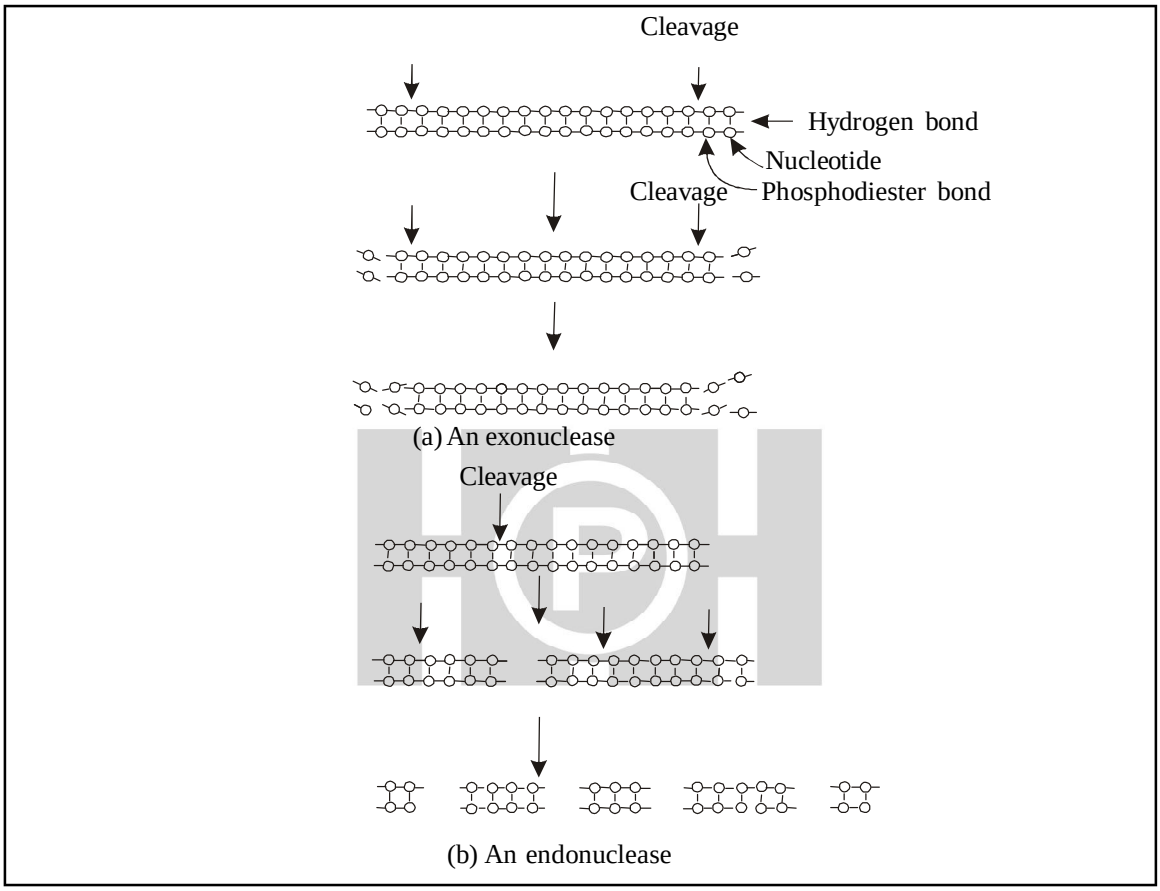


Fig. 1.3: (a) an exonuclease which cuts from the end of DNA, (b) an endonuclease which removes with in the DNA strand

(a) Exonucleases that nucleotides one at a time from the end of a DNA molecule, e.g., BAL31, exonucleaseIII.

(b) Endonucleases are able to break internal phosphodiester bonds, e.g., Sl endonuclease, deoxyribonuclease (DNase I).

EXONUCLEASES

The difference among different exonucleases lies in the number of strands that are degraded, when a double-stranded molecule is attacked. The enzyme called BAL31 (from the bacterium *Alteromonas espejiana)* is an example of an exonuclease that removes nucleotides

from both strands of double-stranded molecule (Fig. 1.4). The more the length for which BAL31 is allowed to act on a group of DNA molecules, the shorter the resulting DNA fragments will be. However, enzymes such as *E.coli* exonuclease will degrade just one strand of a double-stranded molecule, leaving single-stranded DNA as the product.

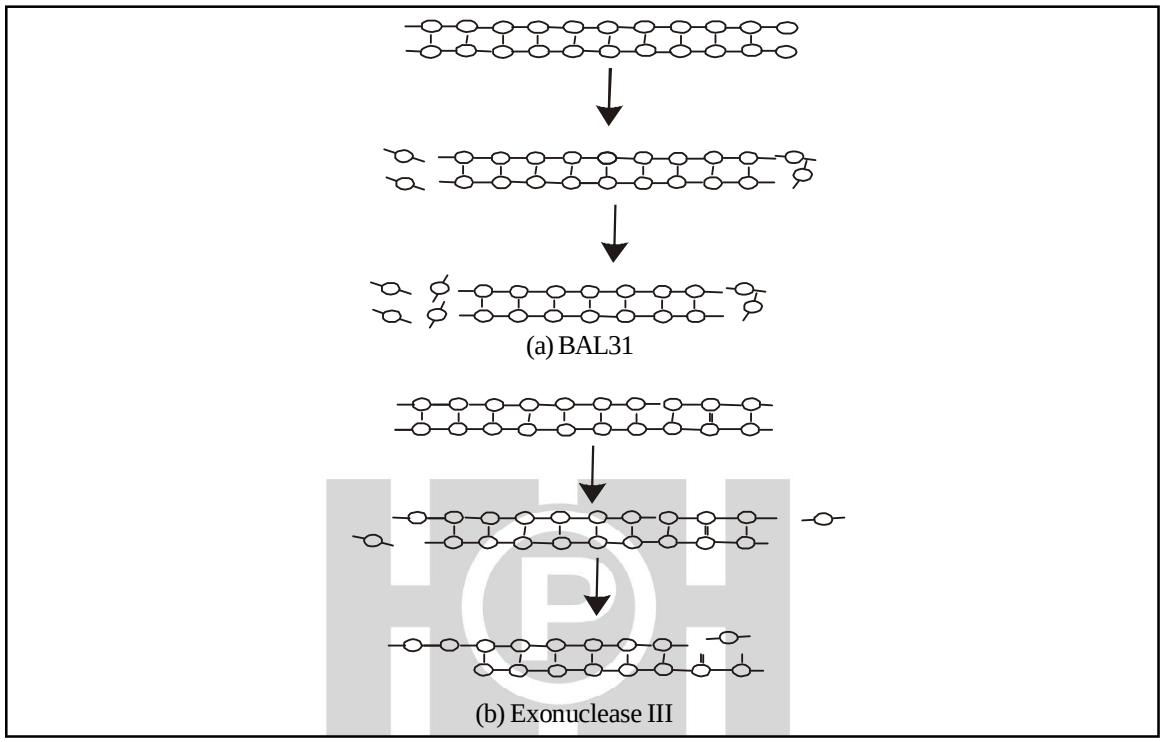


Fig. 1.4: The reactions catalyzed by different types of exonuclease. (a) BAL31, which removes nucleotides from both strands of a double-stranded molecule. (b) exonuclease III, which removes nucleotides only from the 3'-terminus.

ENDONUCLEASES

Endonuclease (from the fungus *Aspergillus oryzae*) only cleaves single strands (Fig. 1.4) whereas deoxyribonuclease I (DNase I), which is prepared from cow pancreas, cuts both single and double-stranded molecules (Fig. 1.4). DNase I is non-specific in that it cuts DNA at any internal phosphodiester bond and therefore DNase produces a mixture of mononucleotides and very short oligonucleotides.

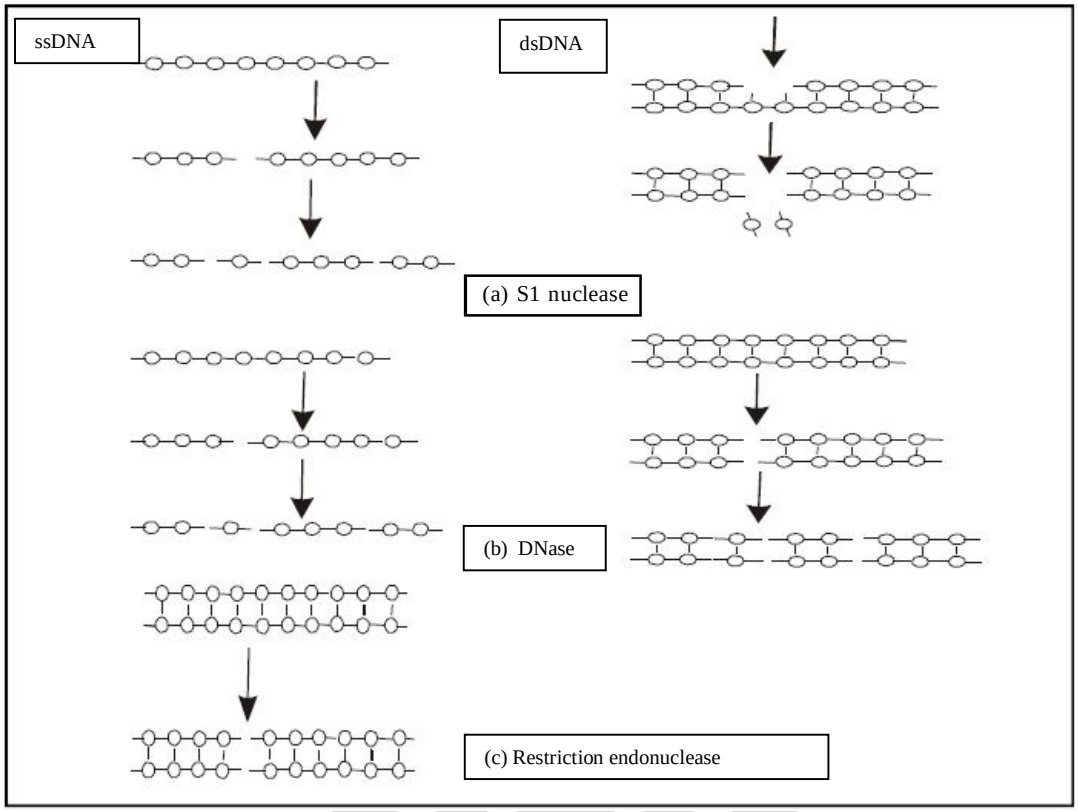


Fig. 1.5: Nuclease: *(a)* S1 nuclease, which cleaves only single-stranded DNA including single-stranded nicks mainly in double-stranded molecules. *(b)* DNAse I, which cleaves both single- and double-stranded DNA. *(c)* A restriction endonuclease, which cleaves double-stranded DNA, but only at a limited number of sites.

The other group of enzymes called restriction endonucleases cleave double-stranded DNA only at specific sites having certain recognition sequences.

EXONUCLEASES

(a) 3'-5 exonuclease: E.coli exonuclease III

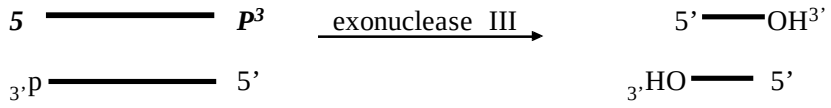
This is a monomeric multifunctional enzyme that removes 5'-mononucleotides from the 3'-hydroxyl end leaving protruding 5'-termini. The second activity is the DNA 3'-phosphatase activity which hydrolyses phosphatase. Third activity degrades the RNA strand in DNA-RNA heteroduplex thus the RNase H activity. The fourth activity of Exo III is 'an AP-Endonuclease' which cleaves phosphodiester bonds at apurinic or apurimidinic sites.

The exonuclease will not degrade single-stranded DNA with a protruding 3'-terminus. Linear double-stranded DNAs containing nicks or gaps are substrated as depicted below.

(i) Activity: 3'-Exonuclease: This enzyme is active on 3'-hydroxyl termini of double-stranded DNA with blunt ends or with ends or and recessed 3'-termini. 3'-hydroxyl termini at nicks are also substrates. The DNA must contain phosphodiester bond; thioesters are not cleaved.

(ii) Activity: 3'-Phosphatase: Double- or single-stranded DNA with a 3'-phosphatase terminus; internal phosphodiester bonds are not cleaved.

Reaction:



USES OF EXONUCLEASE III

(i) Generation of nested deletions.

(ii) Site-specific mutagenesis use thiophosphate derivatives of the dNTPs for second-strand synthesis primed by the mutagenic primer. Because thio-containing nucleotides are resistant to hydrolysis by exonuclease III, digestion will stop at the first place the enzyme encounters thio-substituted nucleotide. By controlling the amount of α-thio incorporation, one can more readily obtain sets of resected DNA fragments of the desired size.

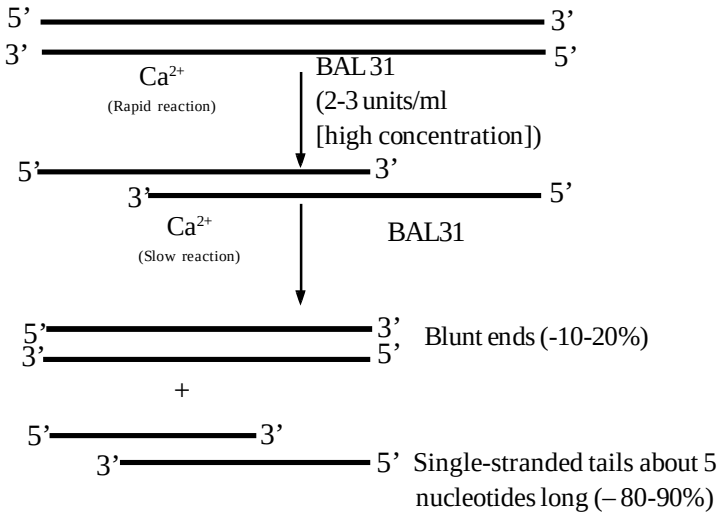
(iii) Exonuclease III is non-processive and typically generates populations of molecules that have been resected to similar extents. This property simplifies the task of isolating DNA molecules whose lengths have been reduced by the desired amount.

(b) BAL31 nuclease

This is a 3'-exonuclease and removes nucleotides from both 3'-termini of the two strands of linear DNA. It is also an endonuclease, therefore, the single-stranded DNA generated by 3'-exonuclease activity is further degraded by the endonuclease. BAL31 isolated from the marine bacterium, *Alteromonas espejiana* sp., is a calcium-dependent nuclease. It has an additional activity besides the above-mentioned two, a 5'- + 3'-exonuclease activity, that works efficiently on single-stranded DNA. The combination of two exonuclease activities causes both strands of double-stranded DNA to get shortened from both ends. BAL31 will also digest single-stranded RNA and double-stranded RNA, the latter such less efficiently than double-stranded DNA.

(i) Activity: Exonuclease/endonuclease

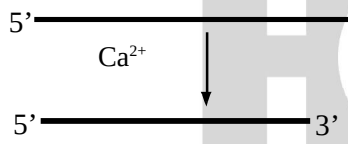
***Substrate*:** BAL31 degrades double-stranded DNA sequentially from both termini. The mechanism is thought to involve a rapid exonucleolytic degradation followed by a slow endonucleolytic reaction on the complementary strand. Double-stranded DNA with blunt or protruding 3'-hydroxyl termini are degraded to shorter double-stranded molecules. The enzyme is also active at nicks, on single-stranded DNA with 3'-hydroxyl termini, and on double-stranded RNA molecules.



(ii) Activity: Exonuclease (shortens single-stranded DNA)

Substrate: Single-stranded DNA with 3'-hydroxy1 termini.

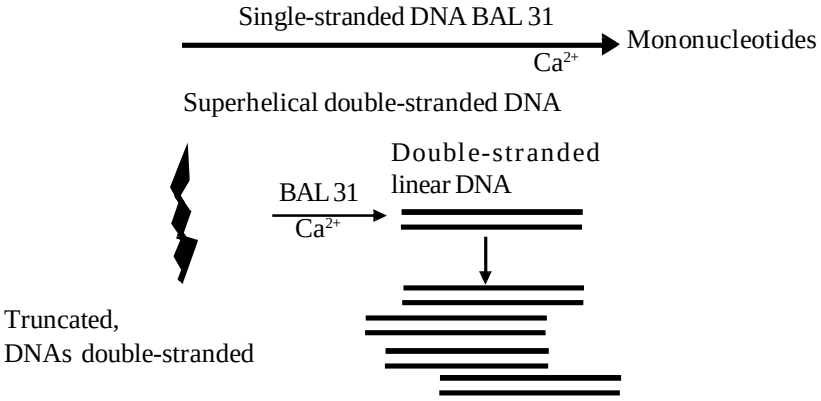
Reaction:



(iii) Activity: Endonuclease

Substrate: Single-stranded DNA; DNA with B-DNA, Z-DNA junctions and other non-B-DNA conformations.

Reactions:



Restriction Endonucleases (RE):

History

RE were initially discovered in the early 1950's as a part of restriction (R) and modification (M) systems that bacteria operate for their protection against invading bacteriophages.

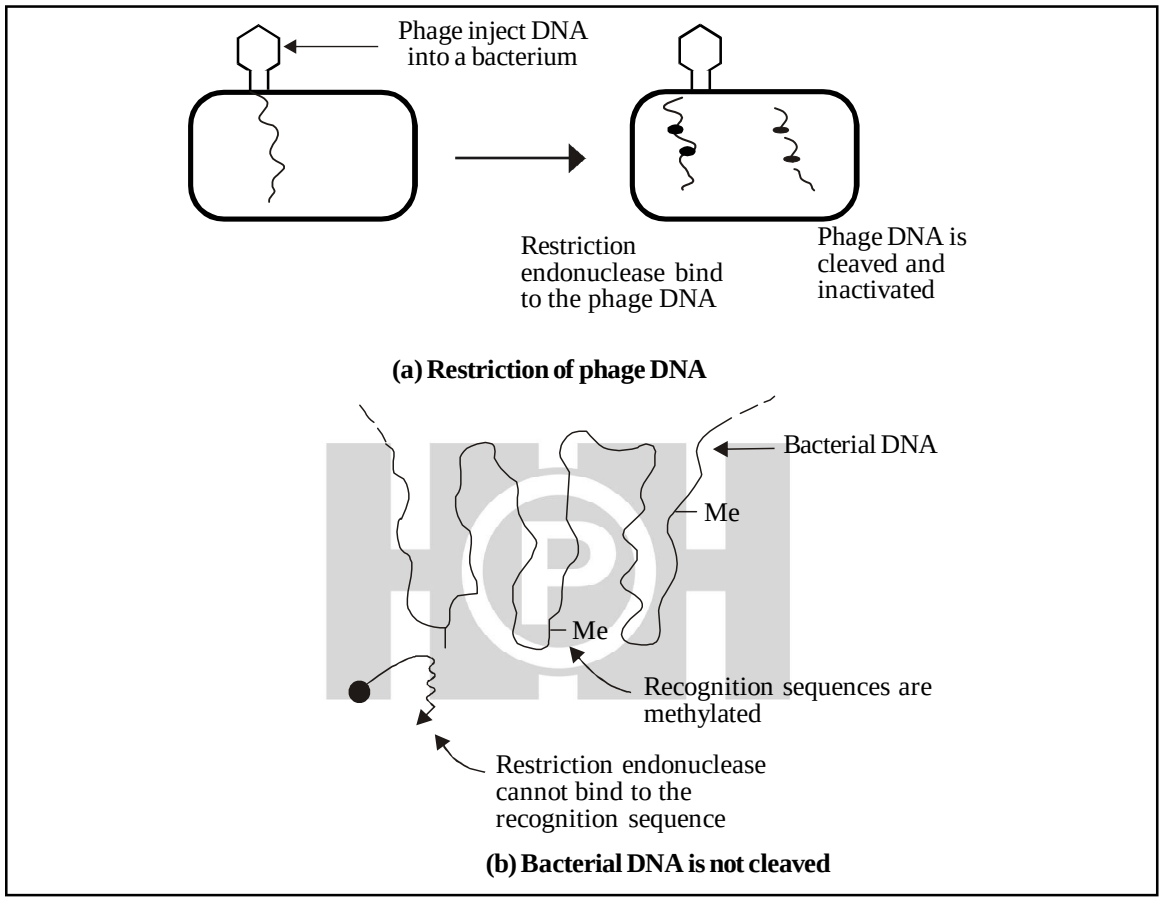


Fig. 1.6: The function of a restriction endonuclease in a bacterial cell: (a) phage DNA is cleaved, but (b) bacterial DNA is not.

RM systems consist of two enzymatic activities: *(a)* a site-specific "restriction" endonuclease (RE) that is responsible for digesting exogenous DNA and *(b)* a DNA "modification" methylase (or methy-transferase) with identical sequences specificity which is responsible for modifying and protecting endogenous DNA from similar digestions by RE.

The preparation of restriction and modification can be explained by the behavior of phage λ on two *E.coli* host strains.

If a preparation of phage is prepared by growth upon *E.coli* strain C and this stock is then titred upon *E.coli* C and *E.coli* K, the efficiency of growth of phage is bigger by several orders of magnitude on *E.coli* C than that on *E.coli* K. The phage is said to be restricted by the second

host strain *(E.coli* K). When those *phage* that do grow on *E.coli* K are now used to reinfect *E.coli* K, they are no longer restricted, but if they are again first grown on *E.coli* C then on *E.coli* K, they are once again restricted. Thus, the efficiency with which phage plates upon a particular host strain depends upon the strain on which it was last propagated. This non-inheritable change brought about in phage by the second host strain *(E.coli* K) that allows it be replated (grown) on that strain without further restriction is called modification. Restriction occurs because the bacterium produces an enzyme that degrades phage DNA before it has time to replicate and direct synthesis of new phage particles. The bacterium's own DNA, the destruction of which would of course be lethal is protected from attack because it carries' additional methyl groups; positioned and added by methyl transferases, that block the degradative enzyme action of restriction endonuclease.

This explains why phage that survive one cycle of growth upon the restrictive host can subsequently reinfect that host efficiently; their DNA bas been replicated in the presence of modifying methylase and so it like the host DNA becomes methylated and protected from the restriction system.

WHAT ARE RESTRICTION ENDONUCLEASES (RE)?

These are the enzymes which break the strands of DNA at internal portions but in a specific way. The site where the RE attached are called as Restriction sites which may range from 4-8 nucleotides. These RE cleave the DNA by cleaving two phosphodiester bonds within each strands of DNA.

Naming of Restriction Endonuclease(RE)

As per the proposal given by Smith and Nathan in 1973, the RE can be nomeclatured as per the following:

1. Every enzyme is named in 3 letter code.
2. The first letter of the code is derived from first alphabet of source (genus).
3. The second and third letter are from species.

Restriction enzyme can be classified (Table 1.1) in the 3 different classes as per their features as given below:

Type I RE: These RE interact with unmodified target site in dsDNA. After traveling long distance between 1000 to 2000 nucleotides, the RE cleaves only one strand. This enzyme is not of much used in rDNA technology.

Type II RE: These are mostly used enzymes in genetic engineering. These RE are highly specific and cleave within or very near to the recognition sequence.

Type III RE: These enzyme cleave dsDNA at defined positions and need ATP, Mg^{2+}.

Table 1.1: Feaures of Restriction Endonucleases

	Type I	Type II	Type III
Retention and modifica- tion activities	Single modification activity	Separate multifunctional enzyme	Separate enzyme with a endonuclease subunit and methylase
Protein structure	3 different sub-units	Simple	2 diferent sub-units
Requirements for restri-ction	ATP, Mg^{2+} S-adenosylmethionine	Mg^{2+}	ATP, Mg^{2+} adenosylmethionine
Sequence of host speci-ficity sites	*EcoB* TGANRTGCT	Rotational symmetry	*E*co Pl: GCC *E*co Pl:5.CAGCAG
Cleavage sites	Possibly random, at least 1000 bp	At or near host specificity site	24-26 bp away from host specificity site
Enzymatic turnover	No	Yes	Yes
DNA translocation	yes	No	No
Site of methylation	Host specificity site	Host specificity site	Host specificity site

Table 1.2: Restriction Enzymes Now in Common Use

Source microorganism	Enzyme	Recognized sequence and cleavage site (↓) ±
Anabaena cylindrica	AcyI	G(Pu) CG(Py)C
Anabaena subcylindrica	AsuII	TT CGAAS
Anabaena variabilis	AvaI	C (Py)CG(Pu)G
	AvaII	G G(A)CC
	AvaIII	ATGCAT
Anabaena variabilis	AvrII	CCTAGG
Arthrobacter leteus	AluI	AG CT
Bacillus amyloliquefaciens	BamHI	G GATCC
Bacillus bravis	BcII	GC*GC
Bacillus caldolyticus	BcII	T GATCA
Bacillus caldolyticus	BcII	T GATCA
Bacillus globigii	BgII	GCC(N)4s↓NGGC
	BgII	A GATCT
Bacillus/stearothermophilus ET	BseEII	G GTNACC
Brevibacterium Jlbidum	Ban	TGG CCA

CaryophJnoll Iatun. L Desulfovibrio desulfuricans, Norway srain	ClaI DdeI	AT CGAT C TNAG
Diplococcus pneumoniae	DpnI	G*A TC
Escherichia coli R Y 13	EcoRI	GAA *TC
Escherichia coli R245	EcoRII	CC*GG
Fusubacterium nucleatum D	fnuDII	CGCG
Haemophilus aegyptius	HaeIII	GG C*sC
Haemophilus aphrophilus	HpaII	ClCGG
Haemophilus gallinarum	HgaI	GACGC
Haemophilus haemolyticus	Hhal	GC*G C
Haemophilus influenzae Rd HindIII	HindII A*AGC↓T	A* AGC↓T
Klebsiella pneumonia. OK8	Kpnl	GGTAC↓C
Micracoleus species	Mstl	TGC GCA
Moraxella bovis	Mbol Mbon	GATC GAAGA
Moraxella nonliquefaciens	MnII	CCTC
Proteus vulgaris	PvuI PvuII	CGAT CG CAG CTG
Providencia stuartii 164	PstI	CGCA G
RhodospiriIium rubrum	RruI	AGT ACT
Thermus aquaticus YTI	Taql	T CGA.
Xantbomonas badrii	Xbal	TCTAGA
Xantbomonas holcicola	Xbol	C TCGAG

Enzymes are named with abbreviations of the bacterial strain, from which they are isolated; the Roman numeral indicates an enzyme's relative order of discovery in that strain (i.e., Acyl was the first restriction enzyme to be isolated from *Anabaena cylindrica)*. *Recognition sequences are written 5' – 3' (only one strand is given and the point of cleavage is indicated by an arrow (↓). For example, G↓GATCC is an abbreviation for:

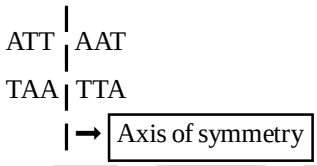
(5')G ↓ GATCC3')

(3')CCTAG↑ G(5')

When no arrow appears, the precise cleavage site has not been determined. The symbol Pu (purine) indicates that either A or G will be recognized; Py (pyrimidine) indicates that either C or T will be recognized. Two bases appearing in parentheses signify that either base may occupy that position in the recognition sequence. Thus, *AvaII* cleaves the sequence GGACC or GGTCC. Where known, the base modified by the corresponding specific methylase is indicated by an asterisk: A. is N-methyladenosine; C is 5-methylcytosine. **(*Source*: R.J. Robens, 1981, *Nuc. Acids Res. 9:75.)***

What are Target Sites?

These are the 4-6 nucleotide long sequences and the DNA which are being recognized by RE Type II and these sequences exhibit palindromic symmetry from central axis, we can read the base as in same orientation as shown below:



Nature of Cuts: Two type cuts are being produced by RE Type II. These are of two types.

(i) Blunt cuts: No free hanging base pairs. These cuts are also known as flush cuts.

(ii) Cohesive cuts: These are also called as staggered cuts, producing free base longs at each end of cleaved DNA.

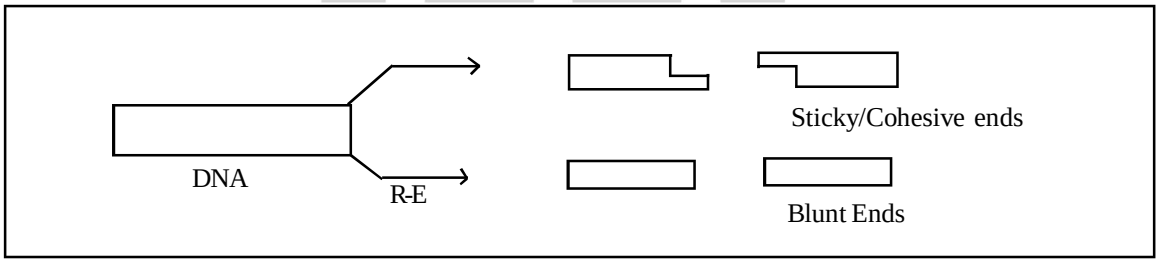


Fig. 1.7: Blunt and cohesive ends upon digestion with Restriction enzyme

Isochizomers: These are the enzymes which are isolated from different sources but recognize same target site., e.g., Asp 718 and KpnI have identical sites.

Role of RE

1. RE are particularly used in Gene cloning experiments to cut insert DNA and vector.
2. RE are used in DNA Restriction digestion during southern blotting in order to detect copy number of Gene.

Star activity: Several enzyme show relaxation in specificity when the optional conditions are being altered. In such cases, nucleases even recognize other alternative base instead of a specific base. Some conditions like High Ionic buffers, High glycerol conch or high even altered Temperature can change the cutting nature of RE.

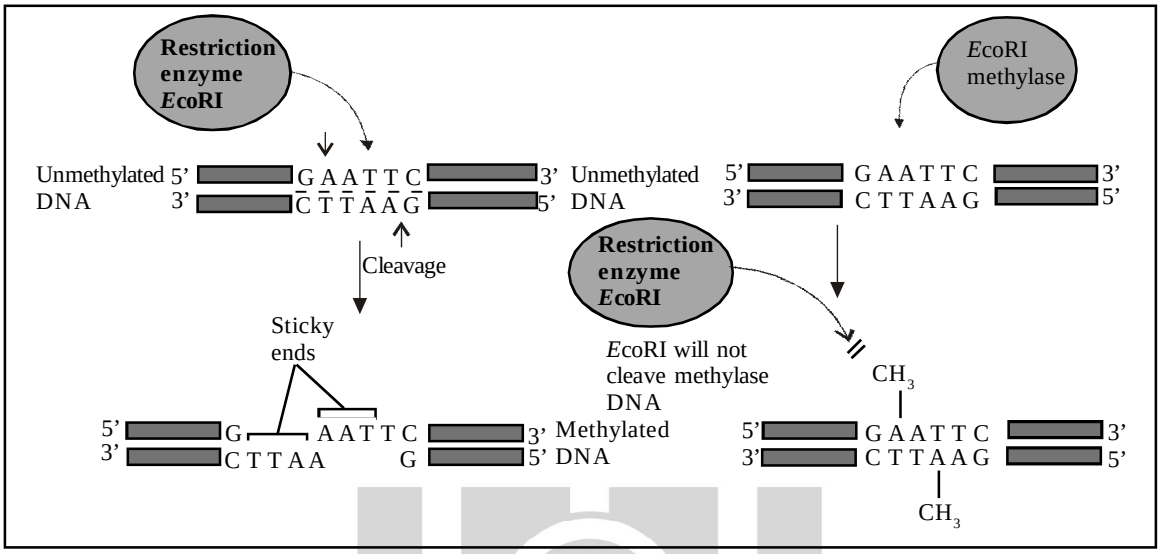


Fig. 1.8: EcoRI and many other restriction enzymes cleave DNA so that the fragments have short complementary single-stranded segments at the ends. These "sticky ends" are important in recombinant DNA techniques because they readily combine with the ends of other cleavage fragments produced by the same restriction enzyme. EcoRI recognizes the six-base-pair sequence. (b) Most restriction enzymes exist in cells along with modification enzyme. The modification enzyme EcoRI methylase catalyzes the methylation of two adenylates *(asterisked)* in the six-base-pair sequence that is normally cleaved by EcoRI. If a methylated sample is then exposed to EcoRI, the added methyl groups protect the restriction site so that the restriction enzyme does not cut the DNA.

Ligases:

In rDNA technology, Ligases are used to join two DNA molecules. These enzymes also play a key role in replication, recombination and cloning. These enzymes are also called as molecular structures.

How Ligases Act?

In the presence of Ligase enzyme when two DNA fragments are mixed, base pairing between two fragments occurs which results in sealing of two different DNA fragments. It occurs due to covalent bonds formation between 2'-PO_4 gp and 3'-OH gp of adjustment strands (Fig. 1.9).

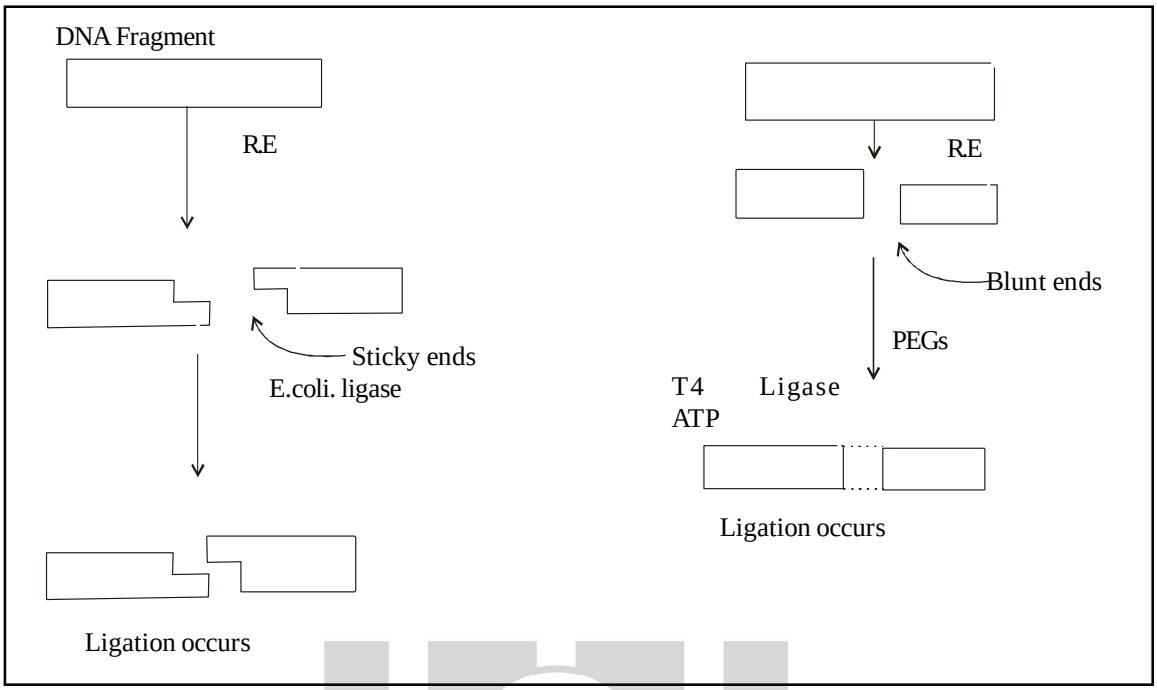


Fig. 1.9: Showing Activity of E.coli and T4 DNA ligases

Two Types of Ligases:

1. E.*coli* ligases which is generally used to join two sticky or cohesive ends.
2. T4 DNA ligase which is generally used to join two blunt-end DNA molecules. All the reactions are generally carried out at 16°C. Sometimes PEG (polyethylene glycol) can be added which acts as volume excluders. PEG increases the frequency of collision between two DNA molecules.

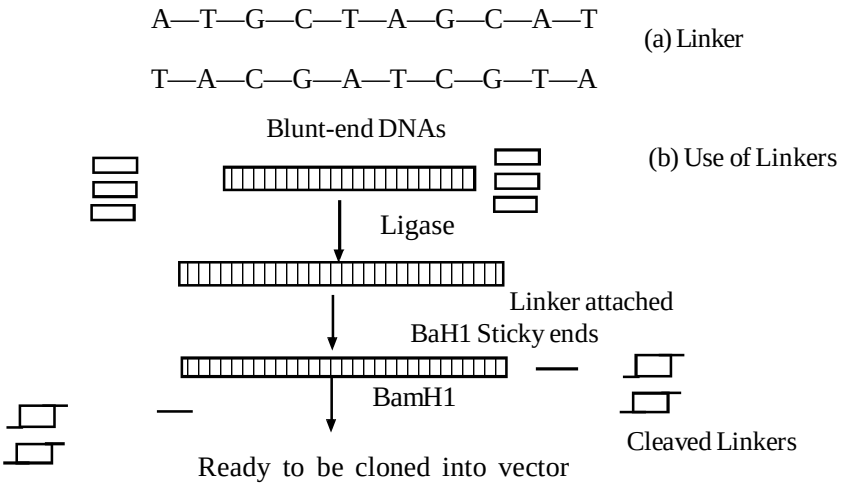
How to put Sticky end to blunt-end DNA?

Sometimes, vector molecule has sticky ends but DNA to be cloned is having blunt ends. So, under these conditions, Linkers and Adaptors play a key role in cloning.

1. Linker: Linker is a short piece of dsDNA of known sequence. It is blunt-ended but contains one restriction site. The structure of a Linker and attachment of Linker to DNA is shown below.

2. Adaptors: One major problem with linkers is that if DNA to be contains one or more BamHI sites, the enzymes will also cut the DNA molecules along with linker molecules. Hence, adaptor molecules have been synthesized as shown below so unlike linkers, an adaptor is synthesized having one end sticky (Fig. 1.10).

Problem with adaptor: Sticky free ends of adaptors can ligase to form dimmers but dimmer formation can be frequented by removing 5'-P group from sticky ends as shown below:



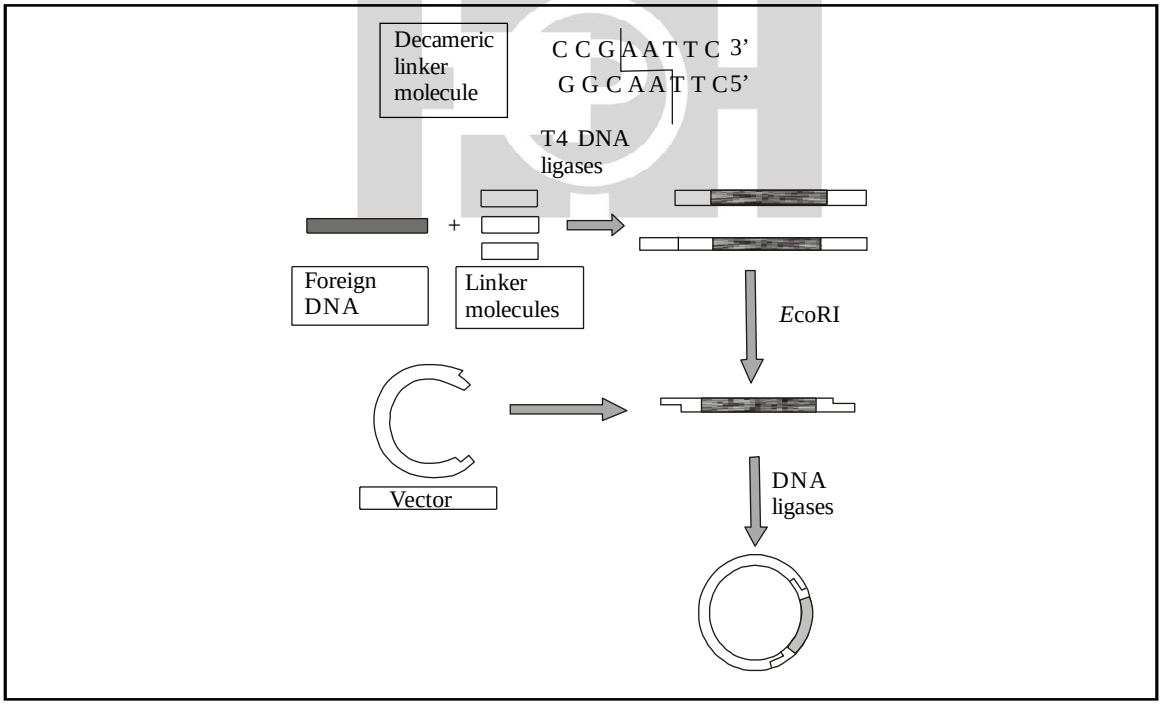


Fig. 1.10: A decameric linker molecule containing an ecoRI target site is joined by T4 DNA ligase to both ends of flush-ended foreign DNA. Cohesive ends are then generated by EcoRI. This DNA can then be incorporated into a vector that has been treated with the same restriction endonuclease.

KINASES AND PHOSPHATASES

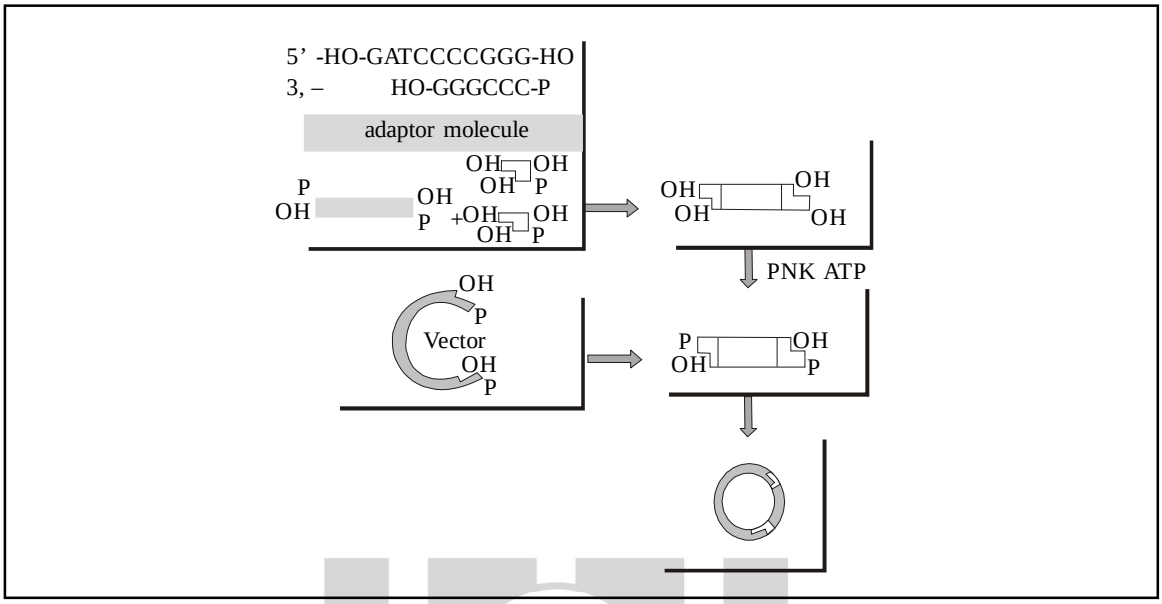


Fig. 1.11: Use of a BamHI adaptor molecule. A synthetic adaptor molecule is lighted to the foreign DNA. The adaptor is used in the 5'-hydroxyl form to prevent self-polymerization. The foreign DNA plus ligated adaptors is phosphorylated at the 5'-termini and ligated into the vector previously cut with BamHI.

OVERVIEW:

Kinases and phosphatases, are common reagents in modern day molecular biology laboratories. Although there are a variety of sources for these enzymes, the most common are calf intestinal alkaline phosphatase (CIAP) and T4 polynucleotide kinase (T4 PNK). Their most frequent use is to modify the phosphorylation state of the 5'-ends of DNA molecules (fig. 1.11). ClAP is most commonly used to remove 5'-phosphates from vector DNA to prevent self-ligation during cloning. Only one strand of a DNA duplex must be joined prior to bacterial transformation; the other will remain nicked until it is repaired inside the bacteria. While the vector DNA is dephosphorylated, the insert DNA should not be dephosphorylated as 5'- phosphates are required for a successful ligation reaction. ClAP is also used to end-label DNA fragments by removing 5'-phosphates, making the DNA fragments better T4 PNK substrates. Synthetic DNA, usually in the form of custom-made oligonucleotides, is devoid of 5'-phosphates and is therefore a less than ideal template for ligation reactions. T4 PNK is routinely used to transfer a λ-phosphate from a nucleotide triphosphate (usually A TP) to the 5'-end of oligonucleotides to facilitate ligation. For blotting, gel-shift or sequencing procedures, [λ-32P]A TP is used as the phosphate donor, resulting in a radiolabeled species. The 5'-end of a DNA molecule generated by restriction endonuclease cleavage can also be labeled, even though a phosphate already exists at that position. This can be achieved either by making use of the exchange activity of T4 PNK to

exchange the existing phosphate with a radiolabeled phosphate from the phosphate donor H, or by first treating the DNA with ClAP to remove the existing phosphates, then adding the radiolabeled phosphate with PNK via the forward reaction, which will result in a high specific activity. Finally, T4 PNK has a 3'-phosphatase activity that can be used to remove phosphate groups from the 3'-terminus of DNA and RNA. Although both enzymes are most commonly used for cloning purposes, they have other activities and are also used for other types of studies. These other activities will be listed in further detail in the following section. The robustness and versatility of ClAP and T4 PNK have made them staples in today's molecular biology applications.

T4 Polynucleotide Kinase

Composed of identical sub-units. The relative mobility of the monomers as measured by SDS-PAGE is 33kDa, by centrifugation; 33.2kDa. From the sequence of T4 PNK, the monomer consists of 301 amino acids with a predicted molecular weight of 34kDa. The molecular weight of the tetramer as estimated by gel filtration is 140kDa, by centrifugation, 147.3kDa. Monomers and dimmers are not enzymatically active.

Description

T4 Polynucleotide Kinase (polynucleotide 5'-hydroxyl-kinase) or ATP: 5'-dephosphopoly-nucleotide 5' phosphatase) or T4 Polynucleotide Kinase (T4 PNK) is a tetramer composed of identical sub-units and has multiple activities. The 5'-kinase activity of T4 PNK catalyzes the transfer of the γ-phosphate from ATP to the 5'-OH terminus of mono- or polynucleotides. The reaction is reversible and in the presence of a nucleotide diphosphate such as ADP the enzyme has 5'-phosphatase activity. Dephosphorylation and subsequent rephosphorylation allow the enzyme to transfer phosphates between A TP and a 5'-phosphate group on an acceptor molecule in an exchange reaction. T4 PNK also has 3'-phosphate activity. T4 PNK can be used to phosphorylate RNA, DNA and synthetic oligonucleotides prior to subsequent manipulations such as ligation. Radioactive phosphate can be used as a label for DNA sequencing, gel shift analysis, footprinting, primer extension, and restriction mapping. Labeling the 5'-ends of DNA and RNA may be done using a dephosphorylated template (5'-OH) using the forward or 5'-kinase reaction. Alternatively, labeling of 5'-ends can be achieved without removal of the existing 5'-phosphate using the exchange reaction. The reaction conditions for the forward and exchange reactions are not the same. The forward reaction generally results in better incorporation. T4 PNK can also be used to remove 3'-phosphatase from DNA and RNA.

Forward Reaction

$$5'—OH—DNA+ [y^{32}—P]ATP \rightarrow P^{32-}O_4—DNA+ADP$$

Exchange Reaction

$$5'—PO—DNA+ADP \rightarrow 5'—OH—DNA+ATP$$

$$\downarrow [y^{32}–P]\ ATP$$

$$5PO^{32}—DNA+ADP$$

Fig. 1.12(a): Describes forward and exchange reactions by kinases

Source: *An E.coli strain that carries a plasmid encoding the modified T4 Polynucleotide Kinase gene.*

Applications

- 5' end-labeling of ss- and dsDNA and RNA
- Phosphorylation of insert DNA prior to ligation.
- Phosphorylation of oligonucleotides.
- Removal of 3'-phosphates 5'-phosphorylation of DNA/RNA for subsequent ligation.
- End-labeling of DNA or RNA.
- 5'-phosphorylation of 3'-phosphorylated mononucleotides to generate a substrate (pNp) that can be added to the 3'-end of DNA or RNA by ligase activity.
- 5'-end labeling of 3'- phosphorylated oligos.

Cofactor Concentration: For the 5'-kinase reaction, 10mM Mg^{2+} optimal at pH 7.6. For the 3'-phosphatase reaction, SmM Mg^{2+} optimal.

Optimal Substrate

5'-Kinase Activity: ss- and dsDNA, ss- and dsRNA, synthetic oligonucleotides and nucleotide 3'-monophosphates. 5'-OH groups on ssDNA overhangs in dsDNA are phosphorylated more efficiently that 5'-OH groups on blunt or 5'-recessed ends. With increased concentration of ATP or enzyme, blunt- and 5'-recessed ends can be completely phosphorylated. Phosphorylation of 5'-OH groups located at nicks in dsDNA is 10-30X slower than for ssDNA. The reaction does not differ significantly for substrates. T4 PNK can also phosphorylate a variety of modified nucleotides and non-nucleotide substrates. Nucleotides (adenosine), nucleotide 2'-phosphates, 3'-termini, or 5'-termini bearing phosphomonoesterase are not substrates.

Exchange Reaction: ss-and dsDNA, 5'-overhangs of dsDNA and single-stranded oligonucleotides are more efficient substrates than 5'-recessed or blunt ends. 5'-recessed and blunt ends are labeled 15-25% as efficiently as 5'-overhangs. 5'-phosphatase groups at nicks are the most

difficult to exchange (30X less efficient than 5'-overhangs). Some tRNA species can act as substrates for phosphatase exchange.

Stimulators

5'-Kinase Activity: Spermidine (1.7mM optimal) can increase the rate of the reaction threefold (24). Spermidine promotes tetramer formation. Salts such as NaCl, KCL and CsCL (125mM optimal) can increase activity up to 5X. LiCL and NH_4Cl give similar stimulation. This effect is the same for 5'-overhangs on dsDNA and ssDNA, oligo and mononucleotides. Conversely, KCl decreases phosphorylation of 5'-recessed ends and at nicks.

A sulfhydryl compound such as DTT (5mM DTT optimal) is essential for activity. 10mM 2-mercaptoethanol and IOmM glutathione result in 80% and 70% respectively, of the activity observed with DTT. In the absence of a sulfhydryl compound, only 2% of the optimal activity is observed. Several anions (at I 25/lM) are stimulatory, with Cl^-, $Br^- > F^-$, $N0_3^-$, $S0_4^{2-}$. PEG 8000 at 4-6% improves the efficiency of labeling 5'- and 3'-overhangs, blunt ends and at nicks.

Inhibitors

5'-Kinase Activity: Phosphate (Pi) and pyrophosphate (PPi) anions are inhibitors of T4 PNK. Ammonium ions are strong inhibitors of T4 PNK. DNA should not be dissolved in or precipitated from buffers containing ammonium salts prior to treatment with kinase. Sulfate containing polymers (e.g., agar, dextran sulfate and heparin) are inhibitors of T4 PNK. However, addition of cationic compounds such as spermine or polylysine can counteract the inhibition. Nonsulfate polysaccharides have no effect on T4 PNK.

Typical Working Conditions: A TP at ≥ 2-5 fold molar excess over DNA ends results in ≥95% phosphorylation of 5'-overhangs. Increasing concentration of A TP to 100-fold molar excess will allow complete labeling of blunt ends or recessed 5'-ends. Protocols for using T4 PNK are available from Promega.

Storage Conditions: Store at -20°C. T4 Polynucleotide Kinase is supplied in 20mM Tris-HCL (pH 7.5), 25mM KCL, 2mM DTT, 0.1mM EDTA, 0.1mM ATP and 50% (v/v) glycerol.

Unit Definition: One unit is defined as the amount of T4 PNK required to catalyze the transfer of I nanomole of phosphate to the 5'-OH end of a polynucleotide from [λ-32P] A TP in 30 minutes at 37°C. The reaction conditions are: 40mM Tris-HCL (pH 7.5), 10mM $MgCI_2$, 5mM DTT, 0.lmM [λ -32P]A TP, 0.5mM 5'-OH polynucleotide end concentration.

Activity Assays

End Labeling: To test for activity, 5pmol of dephosphorylated primer is incubated with 8 units of T4 PNK for I hour at 37°C in IX Kinase Buffer containing [y-32P]A TP. Following incubation, the amount of [λ-32P]A TP converted to TCA-insoluble material is determined. The minimal passing specification is > 20% incorporation.

Sequencing Assay: This enzyme has been tested and qualified for performance in the TaqTrack ® Sequencing Systems.

Contaminant Assays

Endonuclease Assay: To test for endonucleases activity, 1/lg of supercoiled plasmid DNA is incubated with 25 units of T4 PNK for 5 hours at 37°C in IX Kinase Buffer. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

DNase and RNase Assay: To test for nuclease activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 25 units of T4 Polynucleotide Kinase in IX Kinase Buffer for 3 hours at 37°C, and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is < 3% release for both DNase and RNase.

Calf Intestinal Alkaline Phosphatase E.C.3.1.3.1

Description

Calf Intestinal Alkaline Phosphatase is a phosphomonoesterase that catalyzes the hydrolysis of 5'- and 3'-phosphate of DNA, RNA. ClAP is primarily used to dephosphorylate vector DNA prior to cloning and for removal of 5'-phosphates from RNA and DNA before labeling with [‘λ-32P]NTP and T4 polynucleotide kinase. ClAP has also been widely utilized to dephosphorylate proteins. In contrast to acid phosphatases, ClAP can be utilized for *in vitro* dephosphorylation of proteins under conditions that do not denature the substrate protein. ClAP effectively dephosphorylates proteins containing phosphoserine, phosphothreonine and phosphotyrosine, although it can show preferential dephosphorylation of phosphotyrosine under certain conditions.

The process of removing the phosphate group is called *dephosphorylation.* As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphatase.

Bacterial

In bacteria, alkaline phosphatase is located in the periplasmic space, external to the cell membrane. Since this space is much more subject to environmental variation than the actual interior of the cell, bacterial alkaline phosphatase is comparatively resistant to inactivation, denaturation, and degradation, and also has a higher rate of activity. Although the actual purpose of the enzyme is still not fully understood, the simple hypothesis, that it is a means for the bacteria to generate free phosphate groups for uptake and use is supported by the fact that alkaline phosphatase is usually only produced by the bacteria during phosphate starvation and not when phosphate is plentiful. However, other possibilities exist; for instance, the presence of phosphate groups usually prevents organic molecules from passing through the membrane,

therefore dephosphorylating them may be important for bacterial uptake of organic compounds in the wild. Some complexities of bacterial regulation and metabolism suggest that other more subtle purposes for the enzyme may also play a role for the cell. In the laboratory, however. mutant *Escherichia coli* lacking alkaline phosphatase survive quite well, as do mutants unable to shut off alkaline phosphatase production.

The optimal pH for the activity of the *E.coli* enzyme is 8.0 while the bovine enzyme optimum pH is slightly higher at 8.5.

Uses in Research

Common alkaline phosphatases used in research include:

- For removing phosphate monoesterase to prevent self-ligation.
- Shrimp alkaline phosphatase (SAP), from a species of Arctic shrimp *(Pandalus borealis)*.
- Calf Intestinal Alkaline Phosphatase (CIP).
- Placental alkaline phosphatase (P ALP) and its C terminally truncated version that lacks the last 24 amino acids (constituting the domain that targets membrane anchoring) — the secreted alkaline phosphatase (SEAP).

Alkaline phosphatase has become a useful tool in molecular biology laboratories, since DNA normally possesses phosphatase groups on the 5'-end. Removing these phosphates prevents the DNA from ligating (the 5'-end attaching to the 3'-end), thereby keeping DNA molecules linear until the next step of the process for which they are being prepared; also, removal of the phosphate groups allows radiolabeling (replacement by radioactive phosphatase groups) in order to measure the presence of the labeled DNA through further steps in the process or experiment. For these purposes, the alkaline phosphatase from shrimp is the most useful, as it is the easiest to inactivate once it has done its job.

Another important use of alkaline phosphatase is as a label for enzyme immunoassays.

One common use in the dairy industry is as a marker of pasteurisation in cows' milk. This molecule is denatured by elevated temperatures found during pasteurisation, and can be tested for via colour change of a para-Nitrophenylphosphate substrate in a buffered solution (Aschaffenburg Mullen Test). Raw milk would typically produce a yellow colouration within a couple of minutes, whereas properly pasteurised milk should show no change. There are of course exceptions to this in the case of heat stable alkaline phophatases produced by some bacteria.

Inhibitors

All mammalian alkaline phosphatases, except placental (P ALP and SEAP), are inhibited by homoarginine and similarly all, except the intestinal and placental ones are blocked by levamisole. Heating for 2 hours at 65°C inactivated most is enzymes except Placental is of forms (P ALP and SEAP).

Applications

- Dephosphorylation of 5'-phosphorylated ends of cloning vectors.(Fig. 1.12b)
- Dephosphorylation of 5'-phosphorylated ends of DNA or RNA tor subsequent labeling with 32P using [λ-32P]NTP and T4 Polynucleotide Kinase.
- Dephosphorylation of proteins.

Source: Calf intestinal mucosa.

Molecular Weight: 68kDa.

Typical Working Conditions: 50mM Tris-HCL (pH 9.3 at 25°C), ImM $MgCL_2$, 0.lmM $ZnCl_2$ and 1mM spermidine.

Storage Conditions: Store at -20°C. ClAP is supplied in storage buffer containing 10mM Tris-HCI (pH 8.0), ImM MgCL2, 0.lmM ZnLI2, 50mM KCL and 50% (v/v) glycerol amount of enzyme required to catalyze the hydrolysis of 1 ~lmol of 4-nitrophenyl phosphate per minute at 37°C in 1M diethanolamine, 10.9mM 4-nitrophenyl phosphate, 0.5mM $MgCl_2$ (pH 9.8).

CONTAMINANT ASSAYS

Endonuclease Assay: 1μg of supercoiled plasmid DNA is incubated with 5 units of CIAP in IX Reaction Buffer for one hour at 37°C. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 5 units of Calf Intestinal Alkaline Phosphatase in IX Reaction Buffer for one hour at 37°C, and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is ≤ 3% release for both DNase and RNase.

Blue/White Cloning Assay: Five micrograms of pGEM®-3Zf(+) Vectore(d) is digested with representative restriction enzymes (leaving 5'-termini, 3'-termini or blunt ends). The termini are treated with 5 units of Calf Intestinal Alkaline Phosphatase for 1 hour at 37°C, kinased and ligated. The religated plasmid is then transformed into JMI09 cells that are plated on X-Gal/ lPTG/Amp plates. A minimum of 200-400 colonies are counted. White colonies result from transformation with ligated plasmids with damaged ends. These white colonies represent the number of false positives expected in a typical cloning experiment. Enzymes that generate overhangs, such as *EeaR* I. *Hind* III or *Kpn* I, must produce fewer than 2% white colonies, and blunt-cutting enzymes, such as *Hinc* II must produce fewer than 5% white colonies (I). Transformation efficiency must be ≥ 1 × 105 cfu/μg DNA.

ANTARCTIC PHOSPHATASE (ANTP)

Antartic phosphatse has been derived from Antarctic strain TAB5 which is a psychrophilic strain being isolated at the Dumont d'Urville antarctic station. Antarctic Phosphatase catalyzes the removal of 5´ phosphate groups from DNA and RNA. Since phosphatase-treated fragments lack the 5´ phosphoryl termini required by ligases, they cannot self-ligate . This property can be used to decrease the vector background in cloning strategies. AnTP is a superior choice because it is 100% heat inactivated in 5 minutes at 65°C; as a result you can proceed directly to the ligation reaction without further purification of vector DNA. Since, neither CIP or Shrimp Alkaline Phosphatase (SAP) can be completely inactivated at 65°C in 30 minutes, it may be necessary to purify the DNA after the phosphatase reaction. It is important to inactivate the phosphatase because residual active phosphatase can cause failure in subsequent ligation/ transformation experiments.

Main features:

- Heat inactivated in 5 minutes at 65°C
- Ligate without purifying vector DNA
- Isolated from a recombinant source
- Removes 5' phosphates from DNA, RNA, rNTPs and dNTPs
- Prevents recircularization of cloning vectors
- Supplied with 10X Reaction Buffer

Source:

An *E. coli* strain that carries the TAB5 AP gene, originally cloned in plasmid pNI, recloned in plasmid pEGTAB7-4.1(new England biolabs)

Applications:

- Removing 5´ phosphates from DNA, RNA, rNTPs and dNTPs
- Preparation of templates for 5´ end labeling
- Prevention of recircularization of cloning vectors
- Removal of dNTPs and pyrophosphate from PCR reactions
- Dephosphorylation of proteins

Some 5'-End Labeling Tips

- The forward reaction is much more efficient than the exchange reaction for labeling of phosphorylated ends.
- The template used should be gel-purified. Contamination by short oligonucleotides or tRNA will lower the efficiency of labeling. Consider the use of glycogen instead of tRNA for efficient precipitation of oligonucleotides.

- Oligonucleotides are synthesized with a 5'-hydroxyl group. They can be labeled directly in the forward reaction without prior dephosphorylation.
- Ammonium ions are strong inhibitors of T4 Polynucleotide Kinase; therefore, DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with T4 PNK.
- The final A TP concentration in the forward reaction should be at least 1μM.
- Equal concentrations of A TP and 5'-ends result in about 50% labeling of oligonucleotides. To obtain higher specific activity, the A TP:oligonucleotide ratio should be increased to 10: 1. Only 10% of the label will be transferred but virtually every oligonucleotide molecule will be labeled (I).
- 0.5 pmol of linear DNA equals Ipmol of 5'-ends. To convert μg DNA to pmol:

For dsDNA:

μg × 106 pg/μg x pmol/660pg × 1/N = pmol

where N is the number of nucleotide pairs and 660pg/pmol is the conversion factor for a nucleotide pair.

For ssDNA:

μg × 106 pg/μg × pmol/330pg × 1/N = pmol

where N is the number of nucleotides and 330pg/pmol is the conversion factor for a single nucleotide.

- Single-stranded DNA and double-stranded DNA containing 5'-overhangs are labeled more efficiently than 3'-overhangs (5'-recessed) or blunt ends. Labeling of blunt or recessed ends can be improved by increasing the concentration of ATP in the reaction or by denaturing the template with heat or NaOH prior to labeling. Alternative protocols, involving the addition of PEG 8000 to the reaction may also be used to increase the labeling efficiency of these templates.

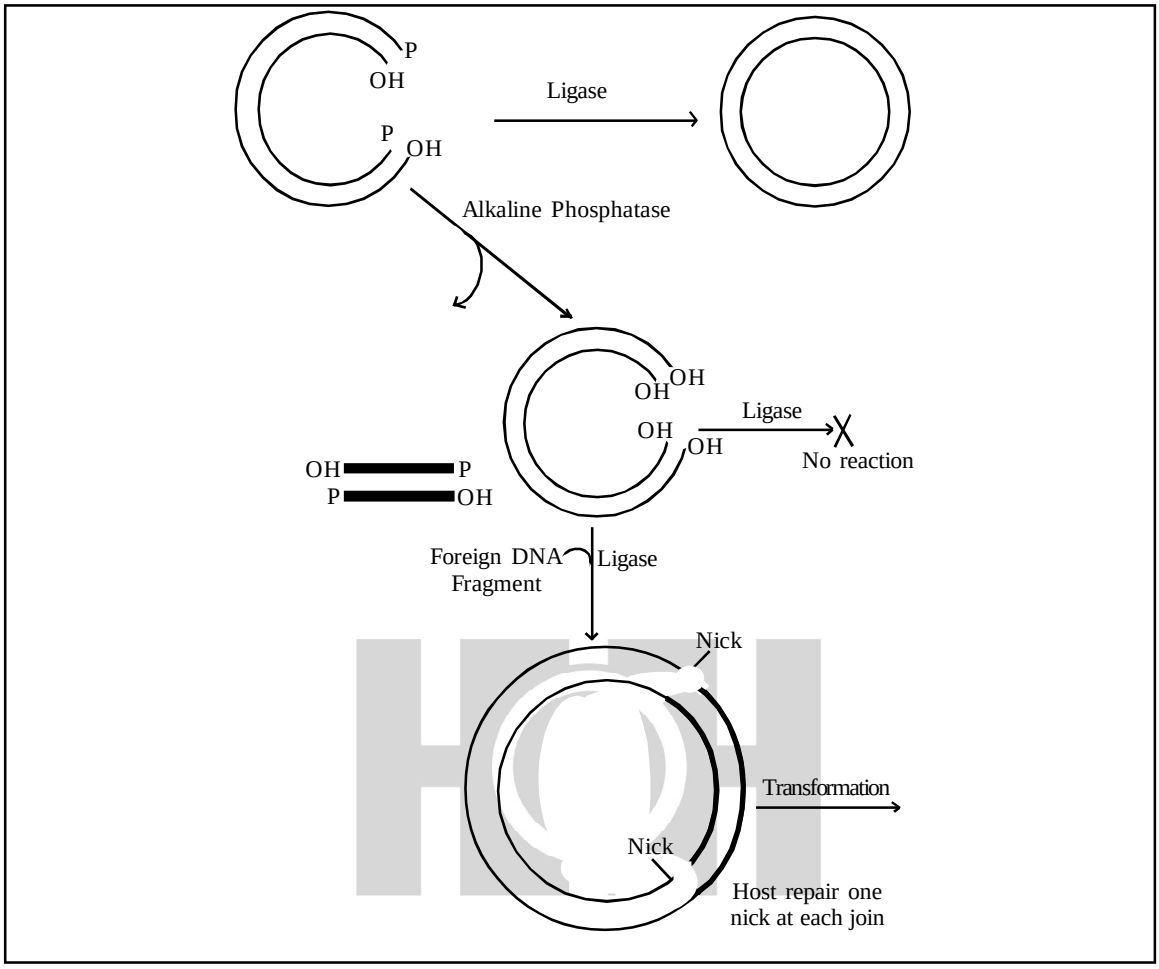


Fig. 1.12(b): Phosphatase treatment to prevent recircularization of vector plasmid without insertion of foreign DNA

NUCLEASE

SI Nuclease is a single-stranded specific endonuclease that cleaves DNA to release 5'-mono and 5'-oligo nucleotides. sDNA, dsRNA and DNA-RNA hybrids are resistant, however, very large amounts of enzyme can completely hydrolyze double-stranded nucleotides. SI nuclease can also cleave single-stranded areas of Superhelical DNA at torsional points where DNA may be unpaired or 'weakly hydrogen bonded'. Once the superhelical DNA is nicked, Sl can cleave the second strand near the nick to generate linear DNA.

SI nuclease is a monomeric protein with molecular weight. It need Zn2+ for its activity, and is relatively stable against denaturing agents such as urea, SDS and formamide. The optimum pH lies between 4-4.5.

Applications of SI Nuclease

1. It is used to analyse DNA-RNA hybrid structures.
2. It can be used to remove single-stranded tails from DNA fragments to produce blunt ends.
3. Hairpin loop structures formed during synthesis of double-stranded cDNA can be digested.

RNASES

Generally, RNase A and RNase TI are used in genetic engineering procedures. Both cleave the phosphodiester bond between adjacent ribonucleotides. However, RNase A cleaves next to uracils and cytosine such that phosphate remains with these pyrimidines. The nucleotide present on the other side of phosphatase is dephosphorylated. RNase A is obtained from the bovine pancreas.

RNase Tl cleaves specifically next to guanine. The phosphatase group at the 3'-end of the nucleotide remains with the cut end. This enzyme is derived from *Aspergillus oryzae*.

RIBONUCLEASE (RNASE H)

RNase H is an endoribonuclease that degrades the RNA portion of the RNA-DNA hybrids. It cuts the RNA into short fragments.

Applications of RNase H

1. RNase H is the key enzyme in the cDNA cloning procedure. Here, it is used to remove the mRNA from the RNA-DNA hybrid.
2. Used to detect the presence of RNA-DNA hybrids.
3. Used to remove poly (A) tails on mRNA.

DEOXYRIBONUCLEASE I

Deoxyribonuclease I (DNase I) is an endonuclease which digest either single-or double-stranded DNA, producing a mixture of mono and oligonucleotides. DNase I hydrolyzes each strand of double-stranded DNA independently and at random. Addition of Mg^{2+}ensures random cleavage while Mn^{2+} gives cleavage nearly at the same place on both strands. The enzyme is derived mostly from bovine pancreas.

Applications of DNase I

This enzyme is useful for a variety of applications including nick translation, DNA footprinting, bisulfite mediated mutagenesis and RNA purification.

Reverse Transcriptase

In the fields of molecular biology and biochemistry, a reverse transcriptase, also known as RNA-dependent DNA polymerase, is a DNA polymerase enzyme that transcribes single-stranded RNA into double-stranded DNA. It also helps in the formation of a double helix DNA once the RNA has been reverse transcribed into a single-stranded cDNA. Normal transcription involves the synthesis of RNA from DNA; hence, reverse transcription is the *reverse* of this. Reverse transcriptase first purified from retrovirus-infected cells, produces a cDNA copy from an mRNA molecule if first provided with an antisense primer (oligo dT or a random primer). This enzyme is critical for converting mRNA into cDNA for purposes of cloning, PCR amplification, or the production of specific probes. Reverse transcriptase was discovered by Howard Temin at the University of Wisconsin-Madison, and independently by David Baltimore in 1970 at MIT. The two shared the 1975 Nobel Prize in Physiology or Medicine with Renato Dulbecco for their discovery.

Well-studied reverse transcriptases include:

- HIV-I reverse transcriptase from human immunodeficiency virus type I (PDB I HMV).
- M-ML V reverse transcriptase from the Moloney murine leukemia virus.
- AMV reverse transcriptase from the avian myeloblastosis virus.
- Telomerase reverse transcriptase that maintains the telomeres of eukaryotic chromosomes.

Terminal Deoxynucleotidyl Transferase (TDT)

It also known as DNA nucleotidylexotransferase (DNTT) or terminal transferase, is a specialized DNA polymerase expressed in immature, pre-B, pre-T lymphoid cells, and acute lymphoblastic leukemia/lymphoma cells. TDT adds N-nucleotides to the variable, diversity, and joining exons during antibody gene recombination. In humans, terminal transferase is encoded by the *DNTT* gene. TDT catalyzes the addition of nucleotides to the 3'-terminus of a DNA molecule. Unlike most DNA polymerases, it does not require a template. The preferred substrate of this enzyme is a 3'-overhang, but it can also add nucleotides to blunt or recessed 3'-ends. Cobalt is a necessary co-factor. However, the enzyme catalyzes reaction upon Mg and Mn administration *in vitro* (Fig. 1.13).

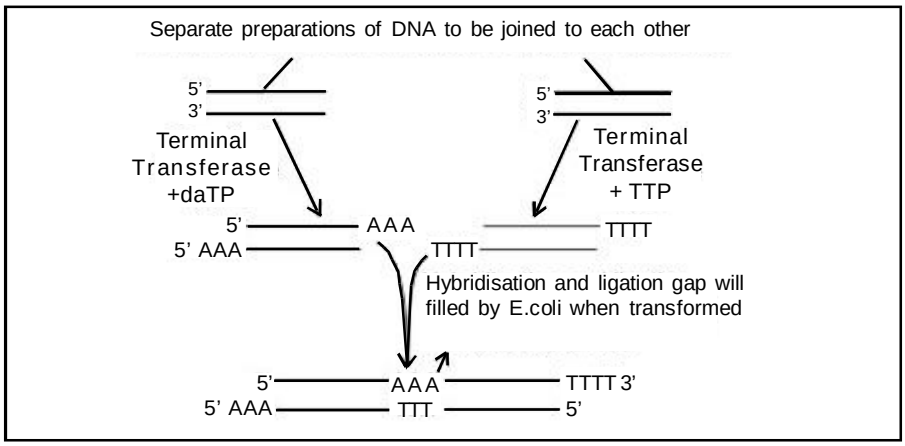


Fig. 1.13

POLYMERASES

DNA polymerase: The enzyme that synthesizes DNA from a DNA template. The intact enzyme purified from bacteria (termed the holoenzyme) has both synthetic and editing functions. The editing function results from nuclease activity.

Klenow fragment: A modified version of bacterial DNA polymerase that has been modified so that only the polymerase function remains; the 5'→3' exonuclease activity has been eliminated.

Thermostabile polymerases: The prototype polymerase, Taq, and newer versions such as Vent and Tth Jolymerase are derived from microorganisms that normally reside at high temperature. Consequently, their DNA polymerase enzymes are quite stable to heat denaturation. making them ideal enzymes for use, in the polymerase chain reaction. It is a thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated hy Thomas D. Brock in 1965. It is often abbreviated to "Taq Pol" (or simply "Taq") and is frequently used in polymerase chain reaction OCR), methods for greatly amplifying short segments of DNA. *T aquaticus* is a bacterium that lives in Hot springs and hvdrothermal vents, and Taq polymerase was identified as an enzyme able to withstand the protein-denaturing conditions (high temperature) required during PCR. Therefore, it replaced the DNA polymerase from *E.coli* originally used in PCR. Taq's optimum temperature for activity is 75-80°C with a half life of 9 minutes at 97.5°C, and can replicate a 1000 basepair strand of DNA in less than 10 seconds. One of Taq's drawbacks is its relatively low replication fidelity. It lacks proof reading activity Hl, and has an error rate measured at about 1 in 9,000 nucleotides. Some thermostable DNA polymerases have been isolated from other thermophilic bacteria and archaea, such as Pfu DNA polymerase, possessing a proof reading activity and are being used instead of (or in combination with) Taq for high-fidelity amplification. Taq makes DNA products that have A (adenine) overhangs at their 3'-ends. This may be useful in TA cloning, whereby a cloning vector (such as a plasmid) is used which has a T (thymine) 3'-overhang, which complements with the A overhang of the PCR product, thus enabling ligation of the PCR product into the plasmid vector.

RNA polymerase II: This enzyme is used by mammalian cells to transcribe structural genes that result in mRNA. The enzyme interacts with a number of other proteins to correctly initiate transcription including a number of general factors, and tissue-specific and induction-specific enhancing proteins.

RNA polymerase III: This enzyme is used by the cell to transcribe ribosomal RNA genes.

Applications of Klenow Fragment

1. In DNA sequencing by sanger method.
2. For production of second strand of cDNA.
3. Radiolabelling by filling in 5'-single stranded extension on double-stranded DNA.

4. "Mutagenesis of" DNA with oligonucleotides.
5. In labelling the DNA by random primer method.

Table 1.3: Active Properties of Some DNA Synthesizing Enzymes

Enzymes	*5'-.3' synthesis*	*5'-Exonuclease*	*3'-Exonuclease*
DNA Polymerase I (E.coli)	✓	✓	✓
Klenow Fragment	✓		✓
DNA Polymerase	✓		✓

✓stands for presence of particular activity by the enzyme. Blank presents absence of such activity.

Table 1.4: Comparative Uses of Some DNA Synthesizing Enzymes

	Nick Trans-lation	*Fill in*	*DNA Sequencing*	*3'-end Labelling*	*Second strand cDNA*	*In Vitro of Mutagenesis*
DNA Polymerase I *(E.col).*	✓	✓		✓		
Klenow Fragment		✓	✓	✓	✓	✓
T-4 DNA Polymerase		✓	✓	✓	✓	✓

✓stands for presence of particular activity by the enzyme. Blank presents absence of such activity.

❋ ❋ ❋