MARUDHAR KESARI JAIN COLLEGE FOR WOMEN (AUTONOMOUS)

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PG and Research Department of Biotechnology

III B.Sc. Biotechnology – Semester - v

E-Notes (Study Material)

Core Course -1: Genetic Engineering	**Code: FBT 51**
Unit: 1 -Tools of genetic engineering - DNA polymerase, poly nucleotidekinase, alkaline phosphates, DNA ligase, nick translation systems, deoxynucleotidyltranserase, reverse transcriptase, restriction endo nucleases. **(14 Hours)**	
Learning Objectives: Mastering the use of these tools in genetic engineering equips students and researchers with the necessary skills to manipulate DNA, study gene function, and develop new biotechnological applications. Understanding the principles, mechanisms, and practical applications of each enzyme is crucial for success in the field of genetic engineering.	
Course Outcome: This course aims to provide comprehensive knowledge and practical skills in the utilization of various enzymes and systems essential for genetic engineering. By the end of this course, students will be proficient in the given areas	

Overview:

- Importance and relevance of the topic.
- **List of main concepts** covered in the topic.

DNA Polymerases

DNA polymerases are a group of enzymes required for DNA synthesis. Arthur Kornberg purified and characterized DNA polymerase from *E.coli* for the first time. It is a single-chain polypeptide now known as DNA polymerase-I. Scientists have now found five DNA polymerases in *E. coli*.

Definition

DNA Polymerases are a group of enzymes that catalyse the synthesis of DNA during replication

DNA Polymerase Function

Replication

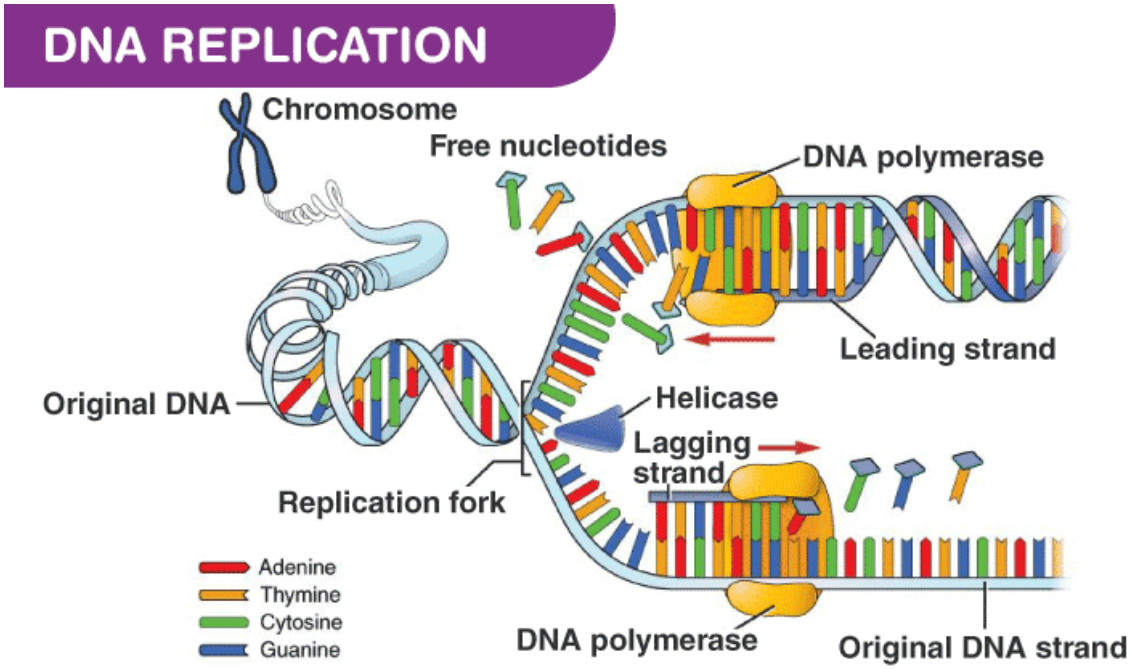
The main function of the DNA polymerase is to synthesize DNA by the process of replication.

It is an important process to maintain and transfer genetic information from one generation to another.

DNA polymerase works in pairs, replicating two strands of DNA in tandem. They add deoxyribonucleotides at the 3′-OH group of the growing DNA strand.

The DNA strand grows in 5’→3’ direction by their polymerisation activity. Adenine pairs with thymine and guanine pairs with cytosine.

DNA polymerases cannot initiate the replication process and they need a primer to add to the nucleotides.



DNA polymerase III is the main enzyme responsible for replication in prokaryotes. In eukaryotes, DNA polymerase **δ** is the main enzyme for replication.

DNA polymerase I removes the RNA primer by 5’→3’ exonuclease activity and replaces the primer by its polymerase activity in the lagging strand.

Repair

The replication process is a humongous task and it is important to maintain the integrity of the genome. Apart from replication errors, DNA repair is the continuous process to rectify any

errors in the genome due to DNA damage. There are various mechanisms by which DNA is repaired.

Proof reading

DNA replication is not perfect and there occurs an error after every 10^4 to 10^5 nucleotides added. Removing the incorrect nucleotide sequence or mismatched nucleotides from the newly synthesised strand is very important for the functionality of proteins, which can even lead to cancer. DNA polymerases remove incorrect pairs by exonuclease activity. They move one step back and remove the mismatched pair by 3'→5' exonuclease activity. This is known as proofreading.

DNA polymerases are also involved in the post-replication DNA repair processes and also in translesion synthesis by which DNA polymerase copies unrepaired part of the DNA blocking the progression of replication.

DNA Polymerase Structure and Types

The structure of most of the DNA polymerases resembles a hand, which is holding active sites. The active site of the enzyme has two parts. At the insertion site, nucleotides are added. After adding, the newly formed base-pair migrates to the post-insertion site.

Prokaryotic DNA Polymerase Types and Function

There are five DNA polymerases identified in *E.coli.* All the DNA polymerases differ in structure, functions and rate of polymerization and processivity.

DNA Polymerase I is coded by polA gene. It is a single polypeptide and has a role in recombination and repair. It has both 5'→3' and 3'→5' exonuclease activity. DNA polymerase I removes the RNA primer from lagging strand by 5'→3' exonuclease activity and also fills the gap.

DNA Polymerase II is coded by polB gene. It is made up of 7 subunits. Its main role is in repair and also a backup of DNA polymerase III. It has 3'→5' exonuclease activity.

DNA Polymerase III is the main enzyme for replication in *E.coli.* It is coded by polC gene. The polymerization and processivity rate is maximum in DNA polymerase III. It also has proofreading 3'→5' exonuclease activity.

DNA polymerase III of *E.coli* is made up of a total of 13 subunits, which comprises 9 different types of subunits.

- It consists of two core domains made up of α, ϵ, and θ subunits. It is attached to the γ complex or clamp-loading complex, which is made up of five subunits, $\tau_2\gamma\delta\delta'$. Additional subunits χ and ψ are attached to the clamp-loading complex. β subunits make two clamps with a dimer each. They increase the processivity of the DNA polymerase III.

DNA Polymerase IV is coded by dinB gene. Its main role is in DNA repair during SOS response, when DNA replication is stalled at the replication fork. DNA polymerase II, IV and V are translesion polymerases.

DNA Polymerase V is also involved in translesion synthesis during SOS response and DNA repair. It is made up of UmuC monomer and UmuD dimer.
Eukaryotic DNA Polymerase Types and Function

Like prokaryotic cells, eukaryotic cells also have many DNA polymerases, which perform different functions, e.g. mitochondrial DNA replication, nuclear DNA replication, etc. The nuclear DNA replication is mainly done by DNA polymerase δ and α. There are at least 15 DNA polymerases identified in human beings.

- DNA polymerase δ – It is the main enzyme for replication in eukaryotes. It also has 3'→5' exonuclease activity for proofreading.
- DNA polymerase α – The main function of DNA polymerase α is to synthesize primers. The smaller subunit has a primase activity. The largest subunit has polymerization activity. It forms a primer for Okazaki fragments, which is then extended by DNA polymerase δ.
- DNA polymerase ϵ – The main function is DNA repair. It removes primers for Okazaki fragments from the lagging strand.
- DNA polymerase γ – It is the main replicative enzyme for mitochondrial DNA.

Poly nucleotidekinase

Polynucleotide kinase (PNK) is an enzyme that plays a crucial role in genetic engineering and molecular biology. It has several important applications, primarily due to its ability to transfer phosphate groups to nucleotides, which is useful in various DNA and RNA manipulations.

Functions and Applications of Polynucleotide Kinase (PNK):

1. **Phosphorylation of 5' Ends:**
    - **Ligation of DNA Fragments:** PNK adds a phosphate group to the 5' ends of DNA and RNA, which is essential for ligation by DNA ligases. This is particularly useful when DNA fragments or oligonucleotides need to be ligated into vectors or joined together.
    - **Restoration of Phosphate Groups:** During certain molecular techniques, DNA fragments may lose their 5' phosphate groups (e.g., after dephosphorylation by alkaline phosphatase). PNK can restore these phosphate groups, making the fragments ligatable again.
2. **End-Labeling of DNA and RNA:**
    - **Radiolabeling and Fluorescent Labeling:** PNK can transfer radioactive or fluorescently labeled phosphate groups to the 5' ends of nucleic acids, creating labeled probes for detection in hybridization experiments, such as Southern or Northern blotting, and for studying nucleic acid-protein interactions.
3. **DNA Repair and Recombination Studies:**
    - **Single-Strand Break Repair:** PNK can be used to repair single-strand breaks in DNA by phosphorylating the 5' ends, preparing them for subsequent repair processes.
    - **Double-Strand Break Repair:** In recombination studies, PNK can prepare DNA ends for the repair of double-strand breaks, facilitating the study of homologous recombination and other repair mechanisms.

Alkaline phosphatase

Alkaline phosphatase (ALP) is a valuable tool in genetic engineering and molecular biology. Its utility stems from its ability to remove phosphate groups from DNA, RNA, and proteins, which has various practical applications in cloning and other molecular techniques.

Applications of Alkaline Phosphatase in Genetic Engineering:

1. **Dephosphorylation of DNA:**
    - **Preventing Self-Ligation:** ALP is commonly used to treat DNA fragments, especially vector DNA, to remove 5'-phosphate groups. This prevents the vector from self-ligating (re-closing without inserting the desired DNA fragment) during the cloning process.
    - **Facilitating Ligation of Insert DNA:** By dephosphorylating vector DNA, only the insert DNA (which retains its phosphate groups) can ligate into the vector, thus increasing the efficiency of cloning.
2. **Preparation of DNA Probes:**
    - **Labeling DNA Probes:** ALP can be used to dephosphorylate the 5' ends of DNA, which can then be re-phosphorylated with radioactive or fluorescently labeled phosphates. This is useful in creating labeled DNA probes for various applications, including hybridization experiments.
3. **Removal of Phosphate Groups from RNA:**
    - **RNA Studies:** Similar to its use with DNA, ALP can dephosphorylate RNA molecules, which is useful in studies involving RNA processing, modification, and labeling.
4. **Protein Analysis:**
    - **Phosphoprotein Studies:** ALP can dephosphorylate proteins, helping to study protein phosphorylation states, which are crucial for understanding signal transduction pathways and protein function.
5. **Gene Cloning and Recombinant DNA Technology:**
    - **Vector Preparation:** In gene cloning, vectors such as plasmids or phages are treated with ALP to prevent the re-circularization of the vector without the desired insert, thereby enhancing the efficiency of obtaining recombinant clones.
    - **Linker Addition:** ALP-treated DNA can be used to add linkers or adaptors to DNA fragments, facilitating the insertion of these fragments into cloning vectors.

DNA Ligase

DNA ligase is an essential enzyme in genetic engineering and molecular biology. It plays a critical role in joining DNA strands together by catalyzing the formation of a phosphodiester bond between the 3'-hydroxyl end of one nucleotide and the 5'-phosphate end of another. This ability to "ligate" DNA makes it indispensable in various molecular cloning and genetic manipulation techniques.

Functions and Applications of DNA Ligase:

1. **Ligation of DNA Fragments:**
    - **Recombinant DNA Technology:** DNA ligase is used to join DNA fragments to create recombinant DNA molecules. This is fundamental in cloning, where a DNA fragment of interest is inserted into a plasmid vector.
    - **Blunt-End and Sticky-End Ligation:** DNA ligase can ligate both blunt-end and sticky-end DNA fragments, although sticky-end ligation is generally more efficient due to complementary base pairing that helps align the ends.
2. **Vector Construction:**
    - **Plasmid Construction:** DNA ligase is used to insert DNA fragments into plasmid vectors, allowing for the propagation of the inserted DNA in bacterial or eukaryotic cells.
    - **Library Construction:** In creating DNA libraries, such as genomic or cDNA libraries, DNA ligase helps insert a diverse range of DNA fragments into vectors for subsequent screening and analysis.
3. **Gene Synthesis:**
    - **Oligonucleotide Assembly:** DNA ligase can join multiple short synthetic oligonucleotides to synthesize longer DNA molecules, enabling the construction of synthetic genes and other DNA constructs.
4. **Repair and Replication:**
    - **DNA Repair:** In vivo, DNA ligase plays a critical role in DNA repair processes, such as sealing nicks in the DNA backbone during replication and after DNA damage repair.
    - **Replication Fork Stabilization:** During DNA replication, DNA ligase seals the Okazaki fragments on the lagging strand, ensuring the continuity of the DNA molecule.

Nick translation

Nick translation is a technique used in genetic engineering and molecular biology to label DNA or to introduce modifications into DNA molecules. It involves the enzymatic introduction of nicks into the DNA strand, followed by the addition of labeled nucleotides to the growing DNA chain at the site of the nick. This process is mediated by DNA polymerase I and DNase I, among other enzymes.

Components and Mechanism of Nick Translation:

1. **Enzymes Involved:**
    - **DNase I:** Introduces single-strand nicks into the DNA.
    - **DNA Polymerase I:** Removes nucleotides from the nicked region (exonuclease activity) and simultaneously adds new nucleotides (polymerase activity) using the intact complementary strand as a template.
2. **Process:**

1. **Nick Introduction:** DNase I introduces random single-strand nicks into the DNA.
    2. **Nucleotide Replacement:** DNA polymerase I binds to the nick and removes nucleotides from the nicked strand using its 5' to 3' exonuclease activity.
    3. **Incorporation of Labeled Nucleotides:** As DNA polymerase I removes nucleotides, it adds new nucleotides to the 3' end of the nick. If labeled nucleotides are present in the reaction mixture, they will be incorporated into the DNA.

Applications of Nick Translation in Genetic Engineering:

1. **DNA Labeling:**
    - **Probe Preparation:** Nick translation is commonly used to label DNA probes with radioactive, fluorescent, or other types of labels. These labeled probes are then used in techniques such as Southern blotting, in situ hybridization, and FISH (fluorescence in situ hybridization).
    - **Genome Mapping:** Labeled probes can hybridize to specific sequences, allowing for the identification and mapping of genes or other genomic regions.
2. **DNA Modification:**

- **Site-Specific Mutagenesis:** By using modified nucleotides, nick translation can introduce specific changes into a DNA sequence, useful for studying gene function and protein interactions.
- **DNA Repair Studies:** Nick translation can be used to study the mechanisms of DNA repair by introducing nicks and observing how repair enzymes interact with the DNA.

3. **Cloning and Sequencing:**
   - **Template Preparation:** Nick translation can be used to prepare DNA templates for sequencing, ensuring that the DNA is in a suitable form for further processing.
   - **Fragmentation and Labeling:** It provides a method to fragment DNA and simultaneously label it, facilitating various downstream applications in cloning and sequencing.

Terminal deoxynucleotidyl

Terminal deoxynucleotidyl transferase (TdT) is a unique DNA polymerase that catalyzes the addition of deoxyribonucleotides to the 3' ends of DNA molecules without the need for a template. This property makes TdT a valuable tool in genetic engineering and molecular biology for various applications, particularly those requiring the addition of nucleotides in a template-independent manner.

Functions and Applications of Terminal Deoxynucleotidyl Transferase (TdT):

1. **Adding Homopolymeric Tails:**
   - **Cloning and Ligation:** TdT can be used to add homopolymeric tails (e.g., poly-A or poly-T) to the 3' ends of DNA fragments. This is useful for facilitating the ligation of DNA fragments with complementary tails, thereby enhancing cloning efficiency.
   - **Blunt-End Ligation:** It can convert blunt-ended DNA fragments into sticky-ended ones by adding specific tails, improving the efficiency of subsequent ligation steps.
2. **DNA Labeling:**
   - **End-Labeling:** TdT can incorporate labeled nucleotides (e.g., radioactive, fluorescent, or biotinylated nucleotides) at the 3' ends of DNA molecules. This

is useful for creating probes for hybridization experiments, such as Southern blotting and fluorescence in situ hybridization (FISH).

- **Creating Labeled Probes:** Labeled DNA fragments can be used to detect complementary sequences in various molecular biology techniques, enhancing the sensitivity and specificity of these assays.

3. **Generation of Random Mutagenesis:**
   - **Introduction of Mutations:** By adding random nucleotides to the 3' ends of DNA, TdT can be used to introduce mutations into a DNA sequence. This approach is useful in creating libraries of mutant genes for directed evolution and functional studies.
4. **Immune System Studies:**
   - **V(D)J Recombination:** TdT plays a natural role in the immune system by adding nucleotides at the junctions of V(D)J gene segments during the generation of antibody diversity. In vitro, TdT can be used to study and manipulate these recombination processes.

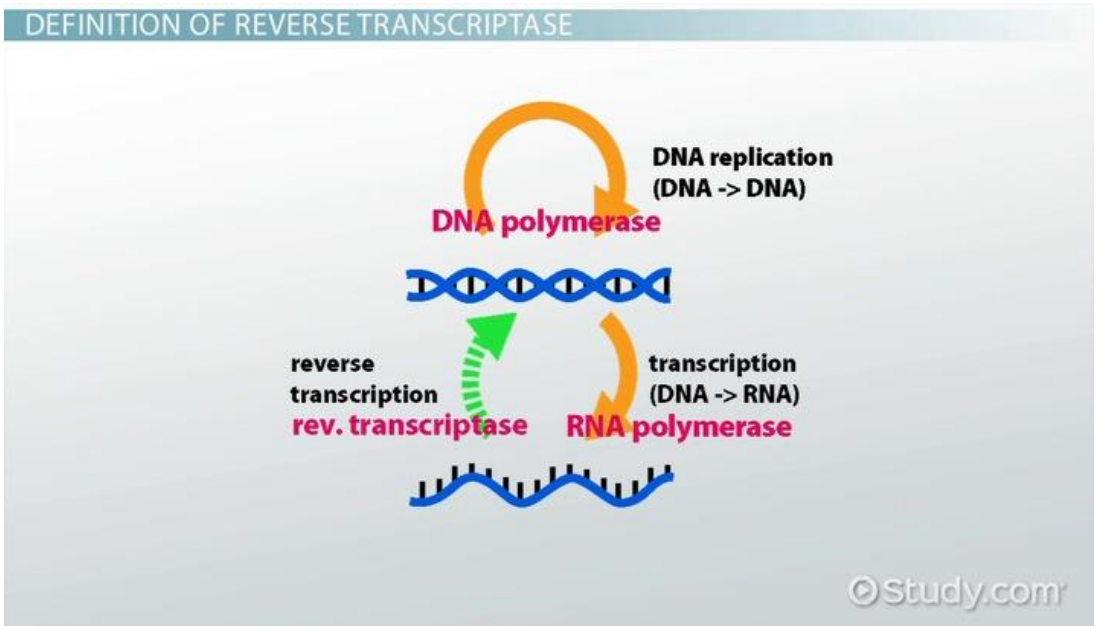
Reverse transcriptase

Reverse transcriptase is an enzyme that catalyzes the transcription of RNA into DNA. This process is the reverse of the usual cellular transcription process, in which DNA is transcribed into RNA. Reverse transcriptase is primarily associated with retroviruses, such as HIV (human immunodeficiency virus).

The key functions of reverse transcriptase include:

1. **RNA-dependent DNA polymerase activity**: The enzyme synthesizes a complementary DNA (cDNA) strand from an RNA template. This is the primary function of reverse transcriptase.
2. **Ribonuclease H (RNase H) activity**: The enzyme degrades the RNA strand of the RNA-DNA hybrid molecule that forms during the initial transcription process.
3. **DNA-dependent DNA polymerase activity**: After degrading the RNA template, the enzyme synthesizes a second DNA strand to form a double-stranded DNA molecule.

These functions allow retroviruses to integrate their genetic material into the host cell's DNA, which is essential for viral replication and propagation.



Restriction endonucleases

Restriction endonucleases, also known as restriction enzymes, are enzymes that cut DNA at specific recognition sites, typically short palindromic sequences. These enzymes are indispensable tools in genetic engineering and molecular biology for manipulating DNA, allowing researchers to cut, modify, and reassemble DNA molecules in a controlled manner.

Functions and Applications of Restriction Endonucleases:

1. **DNA Cloning:**
    - **Cutting DNA at Specific Sites:** Restriction enzymes recognize specific DNA sequences and make cuts, creating defined fragments that can be used in cloning experiments.
    - **Vector Preparation:** Restriction enzymes are used to cut both the DNA of interest and the cloning vector at specific sites, creating compatible ends for ligation.
2. **Genome Mapping:**
    - **Restriction Mapping:** By cutting DNA with different restriction enzymes and analyzing the fragment sizes, researchers can create a map of restriction sites within a DNA molecule, providing insights into its structure and organization.

3. **Recombinant DNA Technology:**
    - **Gene Insertion:** Restriction enzymes are used to insert genes or DNA fragments into vectors for expression in host cells. This process is fundamental in creating genetically modified organisms (GMOs) and producing recombinant proteins.
    - **Fusion Proteins:** Restriction sites can be engineered to facilitate the creation of fusion proteins by combining genes from different sources.
4. **Genomic and cDNA Library Construction:**
    - **Library Preparation:** DNA libraries, including genomic and cDNA libraries, are constructed by cutting DNA into manageable fragments with restriction enzymes and ligating them into vectors for propagation and screening.
5. **Molecular Diagnostics:**
    - **RFLP (Restriction Fragment Length Polymorphism):** This technique involves digesting DNA with restriction enzymes and analyzing the pattern of fragments to detect genetic variations and mutations.
    - **Mutation Detection:** Specific restriction sites can be used to detect mutations by comparing the digestion patterns of normal and mutated DNA.

Reference Book

1. "Molecular Cloning: A Laboratory Manual" by Michael R. Green and Joseph Sambrook
2. “Principles of Gene Manipulation and Genomics" by Sandy B.
3. "Molecular Biology of the Gene**"** by James D. Watson et al
4. "Molecular Biology of the Cell" by Bruce Alberts et al.
5. "Genes IX" by Benjamin Lewin

Additional Link

https://academic.oup.com/book/26881/chapter/195936323

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6137403

Practice Question

1. Explain the role of DNA polymerase in PCR.
2. How do restriction enzymes recognize specific DNA sequences?
3. Describe the function of polynucleotide kinase in molecular cloning.
4. Why is alkaline phosphatase used before inserting foreign DNA into a vector?

5. What is nick translation, and how is it useful in genetic engineering?
6. A researcher wants to join two DNA fragments during cloning. Which enzyme should be used and why?
7. During cDNA library construction, which enzyme is crucial, and what is its function?
8. How can restriction endonucleases be used in forensic science for DNA fingerprinting?
9. If a scientist wants to add nucleotides to the 3' end of a DNA strand, which enzyme would be most suitable?
10. Describe a real-world application of DNA ligase in biotechnology.