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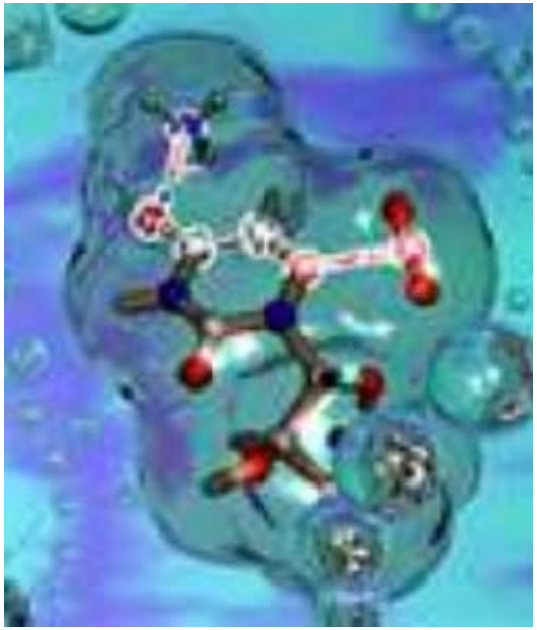

American Society of Cytopathology Core Curriculum in Molecular Biology

Chapter 2

Molecular Science
Biochemical Reagents

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Objectives

- State the function of the following enzymes:
  - Ligase
  - Exonuclease
  - Restriction endonuclease
  - Polymerase
  - Reverse transcriptase

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Introduction

- Enzymes used for nucleic acid modification come from either bacteria or animal tissues
- Common enzymes include those that produce longer polymers, and those that reduce nucleic acids into smaller fragments
- Ability to manipulate nucleic acids in vitro with enzymes has made modern molecular diagnostics what it is today
- Common uses of enzymes:
  - Preparation of nucleic acid samples
  - Labeling of probes
  - Signal generation (example: hybrid capture)
  - Amplification of targets and probes

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Handling of Enzymes in Molecular Laboratory

- Enzymes typically stored at -70°C in a 50% glycerol solution
  - Prevents the enzyme itself from freezing
    - Enzymes are proteins – ice crystals can tear proteins and reduce the quality of enzymatic activity
- For optimal performance of an enzyme:
  - Reaction mixture must mimic source host of the enzyme
    - Buffers, salts, metallic ions, and pH must match those of the organism that the enzyme was isolated from
      - Solution: use buffer mixture provided by manufacturer; typically supplied as a 10X stock
      - Buffer volume is usually 10% of the total; 1/10 dilution of the stock leaves you with a 1X working solution

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Handling of Enzymes (cont.)

- Enzymes must be kept on wet ice while in use in the lab
- After enzyme reaction mix is prepared and enzyme is added to the nucleic acid sample:
  - Store at optimal temp for the enzyme as indicated by the manufacturer's instructions

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Commonly Used Enzymes

- Ligase
- Restriction endonuclease
- Polymerase
- Reverse transcriptase
- Exonuclease

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DNA Ligase

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- Joins two pieces of DNA together
- Forms phosphodiester bonds between existing DNA strandsat the 5' phosphate and 3' hydroxyl group
- Can join two two DNA strands that have cohesive or blunt ends
- Can seal "nicks" in double stranded DNA
- DNA ligase not sequence specific; requires a complementary template

DNA ligase "pastes"or ligates cut fragments of DNA together.

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Nucleases

- Exonucleases: remove bases from the ends of DNA strands
- Endonucleases: cut DNA strands internally

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Exonuclease

- Protects the DNA sequence during replication
- Removes mis-matched bases in DNA sequence
  - Replication errors can result on base changes or mutation in DNA
- Often associated with polymerase enzyme, for the exception of Taq polymerase
- Two principle types of exonucleases:
  - 3' to 5' exonuclease
    - Ensures that replication begins or continues with a correctly base paired nucleotide
  - 5' to 3' exonuclease

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Types of Exonucleases

- Exonuclease I
  - Derived from *E. coli*
  - Degrades single stranded DNA from the 3' hydroxyl end
- Exonuclease III
  - Derived from E. coli
  - Removes 5' mononucleotides from the 3' end of double stranded DNA in the presence of Mg2 and Mn2
  - Removes nucleotides from blunt ends
- Exonuclease VII
  - Digests single stranded DNA from either the 5' phosphate or 3' hydroxyl end
  - One of the few enzymes with 5' exonuclease activity
  - Can be used to remove long single strands protruding from dsDNA
- Nuclease Bal31
  - Derived from Alteromonas espejiani
  - Can degrade single and double stranded DNA from both ends
- Mung bean nuclease
  - Derived from Mung bean sprouts
  - Digests single stranded DNA and RNA
  - Used to remove overhangs from restriction fragments to produce blunt ends for cloning

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Restriction Endonuclease (Restriction Enzyme)

- Originate in bacterial cells
- Bacteria enzymes that are used to protect the bacteria from harmful bacteriophages
- Function: offer protection by cutting DNA into fragments ("endonuclease" – cut into DNA)
  - Cut at very specific sites (hence the term "restriction")
- Restriction endonucleases are named based on the bacteria from which it was isolated
  - Name represents genus and species of the bacteria
  - Roman numeral indicates the sequential isolation of different enzymes

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Restriction Endonucleases

Enzyme	Isolated from	Recognition sequence
Eco RI	*E. coli*, strain R, 1st enzyme	Gv AATTC
Eco RV	*E. coli*, strain R, 5th enzyme	Gv ATATC
Hind III	*H. influenzae*, strain d, 3rd enzyme	Av AGCTT

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Types of Restriction Enzymes

- Type I Restriction Enzymes
  - Possess both nuclease and methylase activity in a single enzyme
  - Binds to host-specific DNA sites of 4-6 base pairs separated by 6-8 base pairs and containing methylated adenines
  - Site of cutting of DNA can be over 1000 base pairs from the binding site
  - EcoK from E. coli K 12 is an example; recognizes the site:
    - 5' – A C N N N N N N G T G C
      T G N N N N N N C A C G - 5'
      - "N" represents non-specific nucleotides and the adenine residues are methylated
- Type III Restriction Enzymes
  - Similar to Type I enzymes in their ability to both methylate and cut DNA
  - Recognition sites are asymmetrical and cutting of DNA occurs 24-26 base pairs from the site to the 3' end
  - *HinfIII* from *H. influenzae* is an example
  - Recognizes the site:
    - 5' – C G A A T
      G C T T A – 5'
      - The adenine methylation occurs on only one strand

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Types of Restriction Enzymes (cont.)

- Type II Enzymes
  - Used most frequently in the laboratory
  - Have been found in almost all prokaryotic cells, but have not been found in eukaryotes
  - Do not have methylation activity
  - Recognition sites are palindromic in nature
    - Read the same 5' to 3' on both strands of the DNA (also known as bilateral symmetry)
  - Cuts DNA directly at the binding site, thus producing fragments of predictable size

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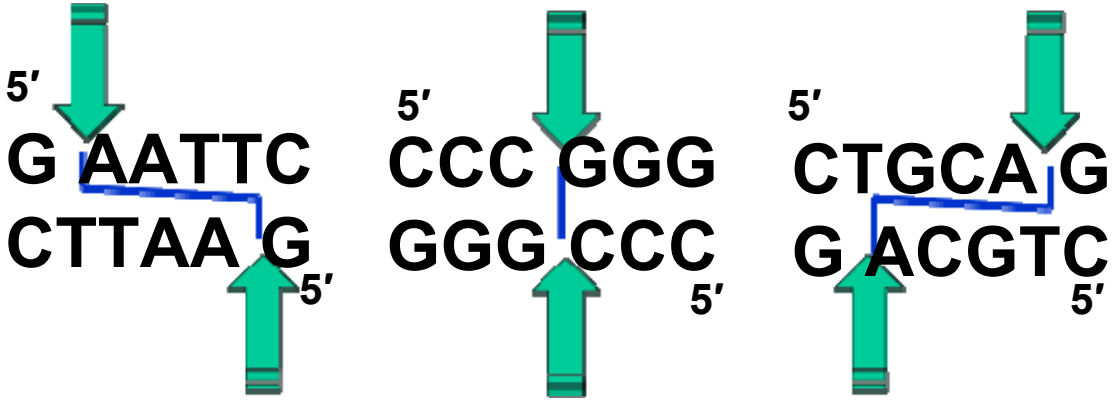
Restriction Endonucleases Cut Double-stranded DNA.



*Ecd*R1
5′ overhang

*Sma*1
blunt

*Pst*I
3′ overhang

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Polymerase

- Catalyzes the synthesis of complementary nucleic acid polymers using a parent strand as the template
- When used in vitro: can extend a primer that is annealed to the template strand (basic principle of PCR, or polymerase chain reaction)
- Extension of a DNA strand requires:
  - The 3'OH groups of the extending end of the strand is free
  - Nucleotide triphosphates are present (dATP, dGTP, dCTP, dTTP)
- Thermostable polymersases are necessary for automated nucleic acid modification
  - Taq polymerase (Thermus aquaticus)
    - Extremely stable at high temperatures

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Reverse Transcriptase

- A polymerase found in retroviruses (a great example would be the HIV virus)
- An RNA-dependent DNA polymerase
- Catalyzes the synthesis of DNA from either an RNA or DNA template
- In vitro reverse transcriptase is used to make complementary DNA (cDNA) copies from an RNA strand

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Summary

- Nucleic acid enzymes occur naturally within living cells. These enzymes can be used in vitro to produced desired genetic material in vitro for use in molecular testing
- Enzymes used in molecular testing should be properly handled at the optimum temperatures to ensure quality test outcomes.

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