

SOS in Biochemistry, Jiwaji University, Gwalior

M.Sc. II Semester (2019-20)

Paper BCH 205: Fundamentals of Molecular Biology (Unit 1)

# ENZYMOMOLOGY *of* DNA REPLICATION - I

# ENZYMOLOGY OF DNA REPLICATION

➤ A variety of proteins is required during the process of DNA replication.

➤ **The Major Proteins are:**

➤ DNA Polymerases

➤ Primase

➤ Ligase

➤ Helicase

➤ Topoisomerase

➤ Gyrase

➤ Single Stranded Binding Proteins (SSBs)

# **DNA synthesis occurs at two places:**

- **Semi-conservative Replication &**
- **DNA Repair Reaction**

**At both places, DNA synthesis is catalyzed by an enzyme**

**i.e., DNA Polymerase**

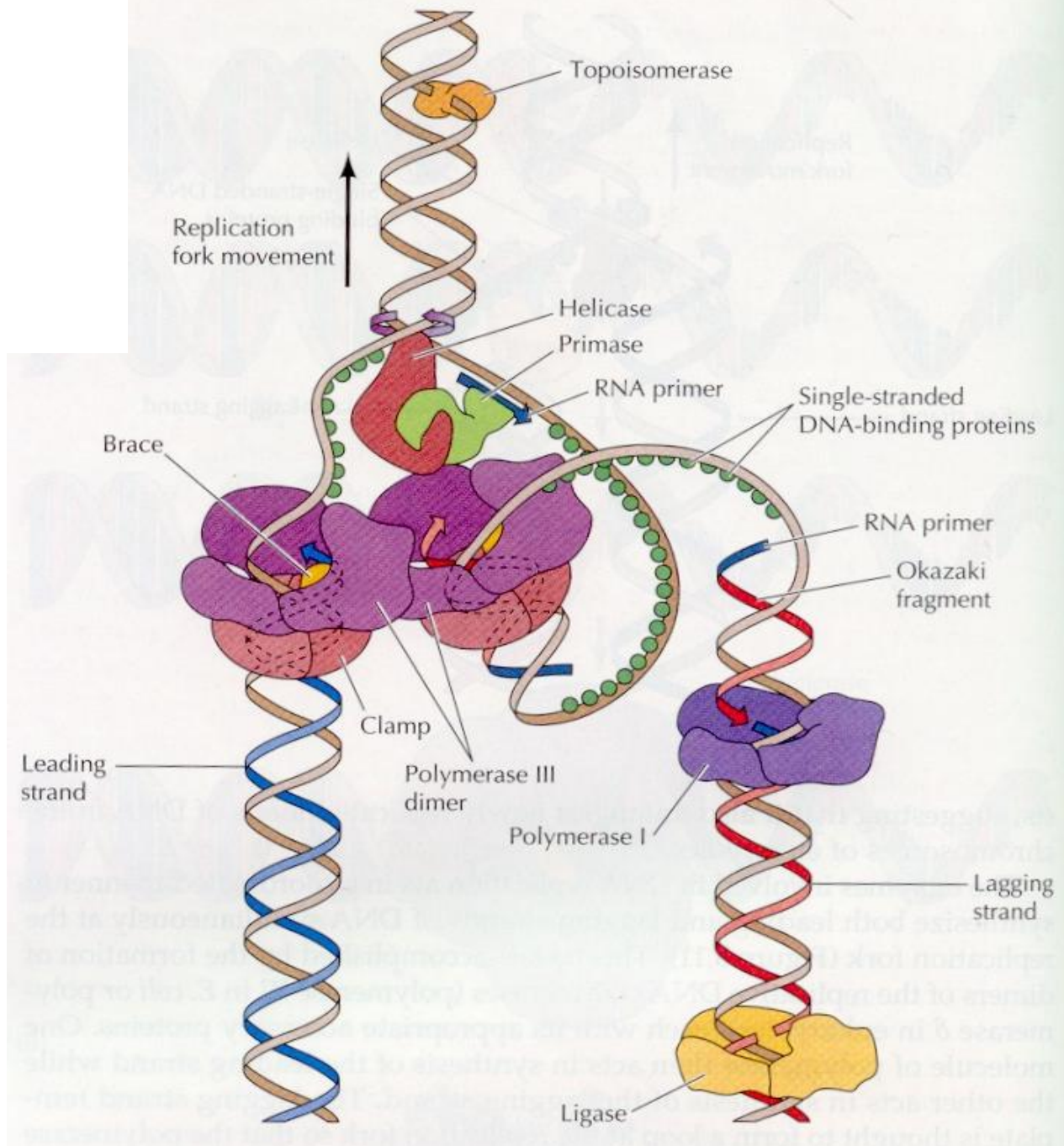
**(or more properly as DNA-dependent DNA Polymerase)**

# We know!!!!!!!!!!!!

1. **Semi-conservative Replication is catalyzed by DNA Polymerase.**
2. **Both prokaryotic and eukaryotic cells contain multiple DNA polymerase activities**
3. **Only few of them actually undertake replication.**
4. **These enzymes are some times called as replicases.**
5. **Both strands are synthesized simultaneously by replicase.**
6. **Different enzyme units are required to synthesize the leading and lagging strands.**
7. **In *E. coli*, both these units contain the same catalytic subunit.**
8. **In other organisms, different catalytic subunits may be required for each strands.**

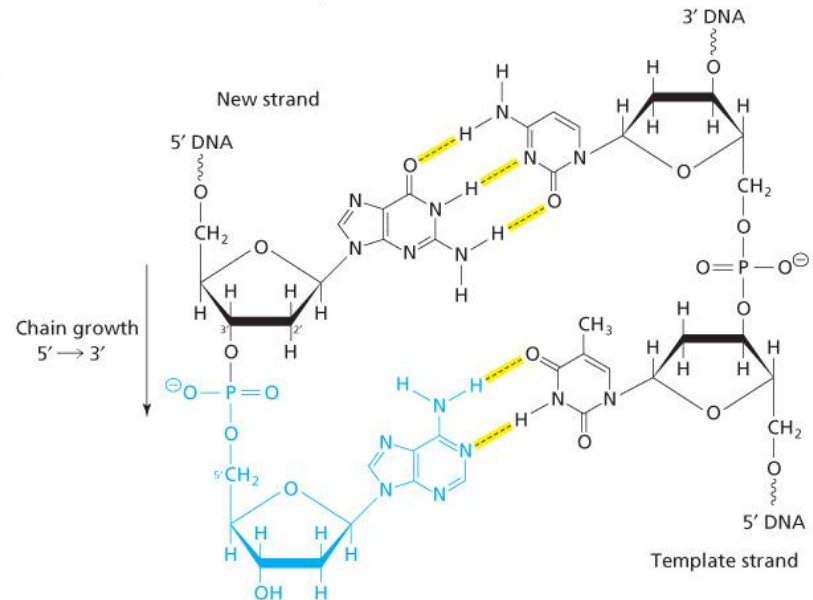
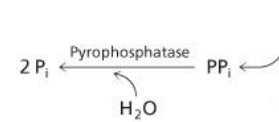
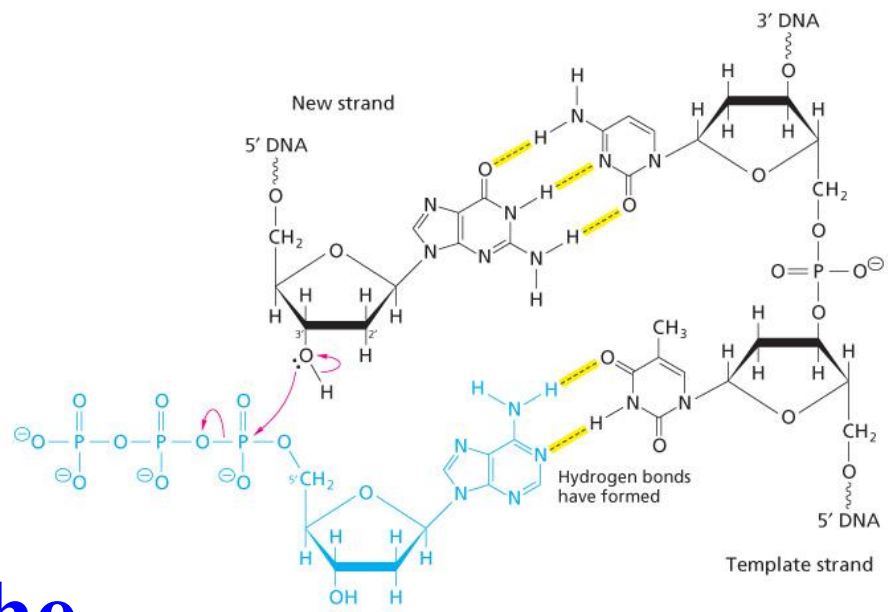
# We know!!!!!!!!!!!!

1. Both prokaryotic and eukaryotic cells contain multiple DNA polymerase activities
2. Main replicating enzyme is called as replicase.
3. Both strands are synthesized simultaneously by replicase.
4. Different enzyme units are required to synthesize the leading and lagging strands.
5. In *E. coli*, both these units contain the same catalytic subunit.
6. In other organisms, different catalytic subunits may be required for each strands.



# Prokaryotic DNA Polymerases

# DNA polymerases are the enzymes that catalyze the attachment of nucleotides to make new DNA





# DNA Polymerases

- In *E. coli* there are five proteins with polymerase activity
  - DNA pol I, II, **III**, IV and V
  - DNA pol I (minor role in replication) and III (major role in replication, replicase)
    - **Normal replication**
  - DNA pol I, II, IV and V
    - DNA repair and replication of damaged DNA

# The DNA Polymerase Family

A total of 5 different DNAPs have been reported in *E. coli*

- **DNAP I: functions in repair and replication** (discovered in 1957)
- **DNAP II: functions in DNA repair** (proven in 1999)
- **DNAP III: principal DNA replication enzyme** (discovered in 1970) by Thomas Kornberg (son of Arthur Kornberg) & Malcolm Gefter
- **DNAP IV: functions in DNA repair** (discovered in 1999)
- **DNAP V: functions in DNA repair** (discovered in 1999)

To date, a total of **14** different DNA polymerases have been reported in eukaryotes

# Arthur Kornberg discovered DNA dependent DNA polymerase

Used an “*in vitro*” system: the classic biochemical approach

1. Grow *E. coli*
2. Lyse cells
3. Prepare extract
4. Fractionate extract
5. Search for DNA polymerase activity using an ASSAY  
(Incorporate radioactive building blocks,  
Precipitate DNA chains (nucleotides soluble),  
Quantify radioactivity.)



**A. Kornberg**



**1959: NP in Medicine & Physiology  
(for synthesis of DNA & RNA)**

Severo Ochoa

# Nobel Prize for DNA polymerase I

Comparison of DNA Polymerases of *E. coli*

	DNA polymerase		
	I	II	III
Structural gene*	<i>polA</i>	<i>polB</i>	<i>polC (dnaE)</i>
Subunits (number of different types)	1	≥4	≥10
$M_r$	103,000	88,000 <sup>†</sup>	830,000
3'→5' Exonuclease (proofreading)	Yes	Yes	Yes
5'→3' Exonuclease	Yes	No	No
Polymerization rate (nucleotides/sec)	16–20	40	250–1,000
Processivity (nucleotides added before polymerase dissociates)	3–200	1,500	≥500,000
Mutant viable?	Yes!	Yes!	No
Function		Repair	Replication

- **DNA pol IV:** error-prone polymerase
  - **DNA pol V:** error-prone polymerase
- (both are involved in allowing replication to bypass certain types of damage)*

# DNA Polymerases

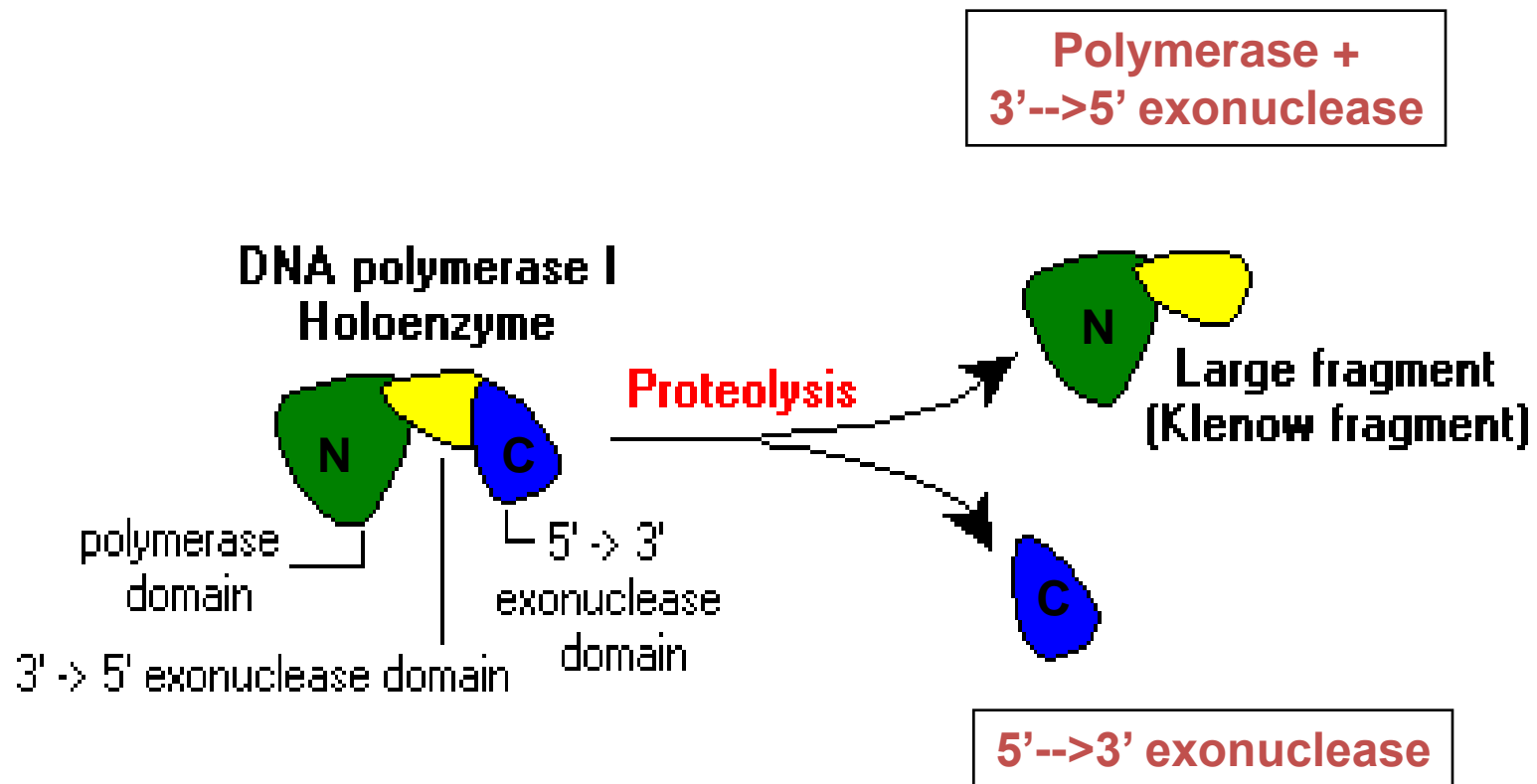
## ■ DNA pol I

- Composed of a single polypeptide
- Removes the RNA primers and replaces them with DNA

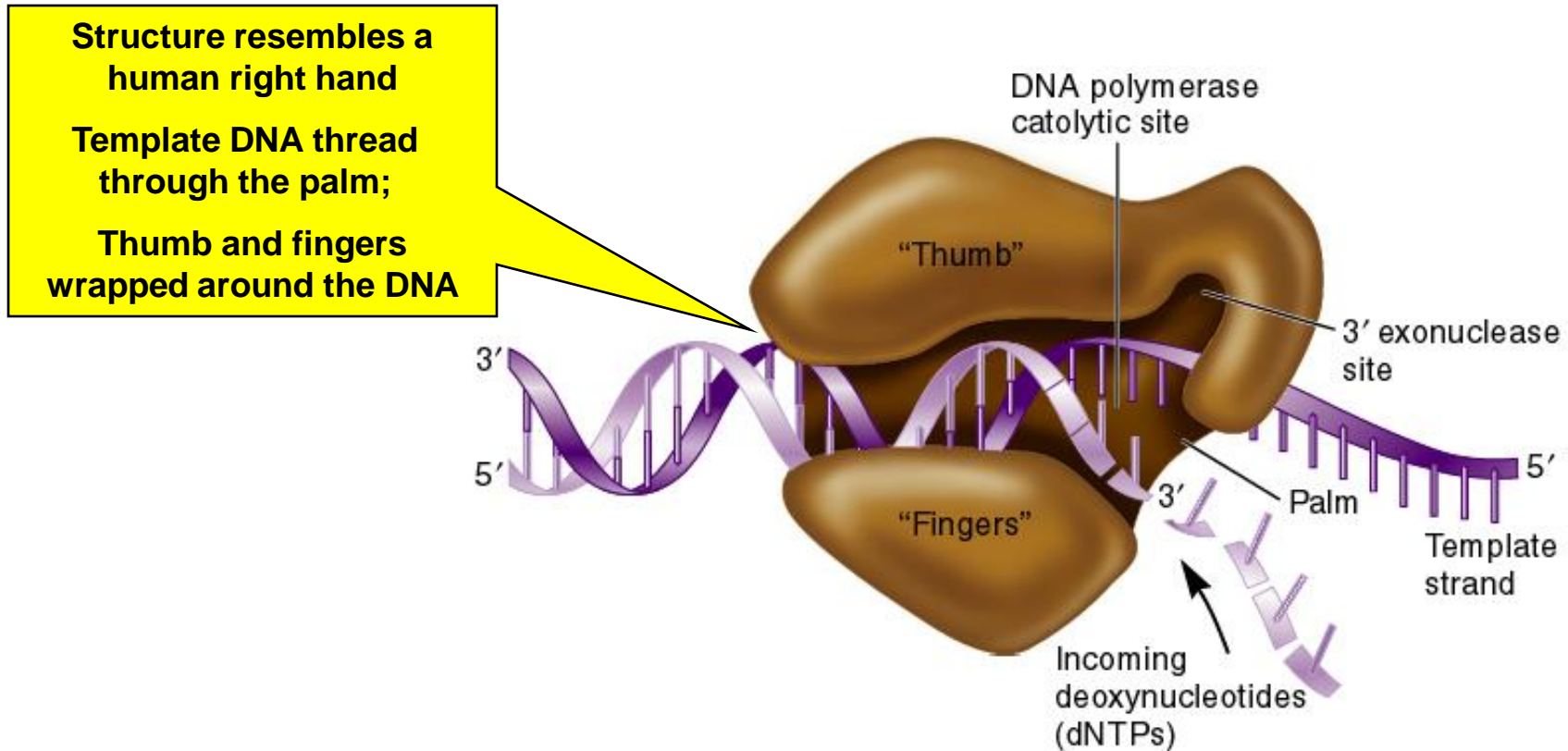
## ■ DNA pol III

- Composed of 10 different subunits
  - The  $\alpha$  subunit synthesizes DNA
  - The other 9 fulfill other functions
- The complex of all 10 is referred to as the **DNA pol III holoenzyme**

# DNA pol I Klenow fragment lacks 5'→3' exonuclease



- Bacterial DNA polymerases may vary in their subunit composition
  - However, they have the same type of catalytic subunit

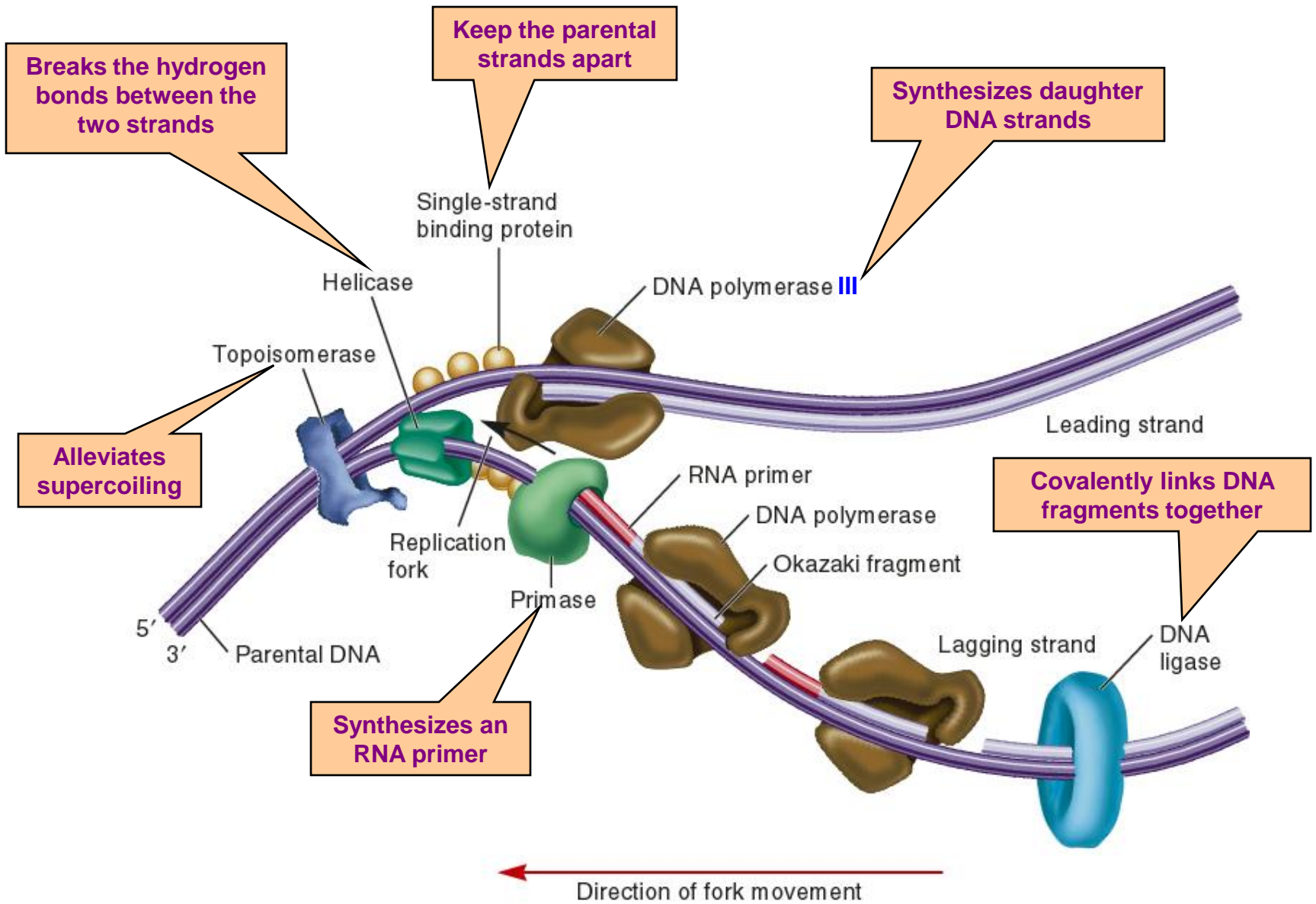


(a) Schematic side view of DNA polymerase

- **DNA pol I** removes the RNA primers and fills the resulting gap with DNA
  - It uses its 5' to 3' exonuclease activity to digest the RNA and its 5' to 3' polymerase activity to replace it with DNA
- After the gap is filled a covalent bond is still missing
- **DNA ligase** catalyzes a phosphodiester bond
  - Thereby connecting the DNA fragments

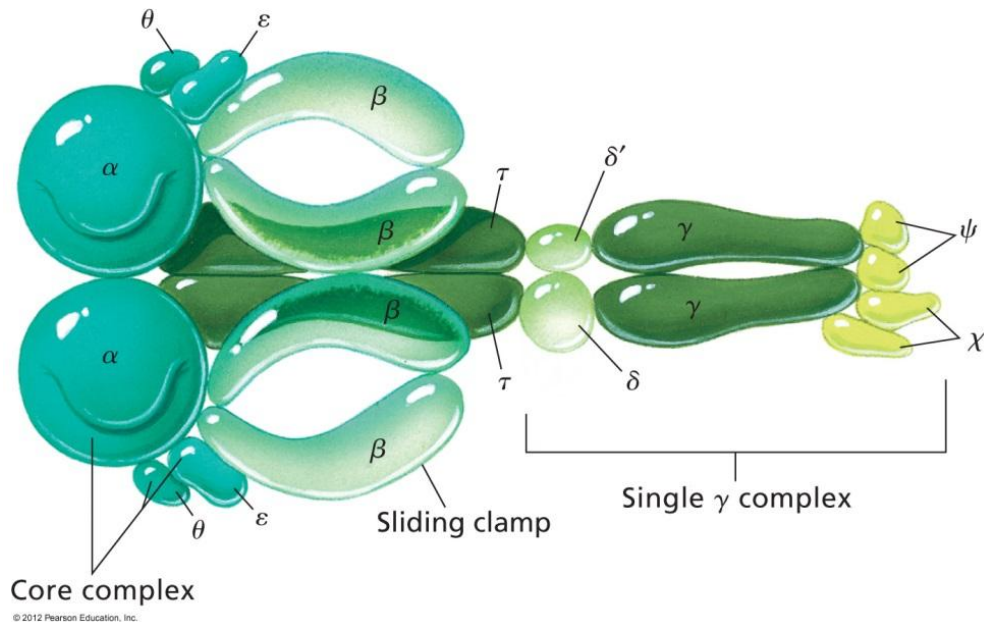


- The two new daughter strands are synthesized in different ways
  - **Leading strand**
    - One RNA primer is made at the origin
    - DNA pol III attaches nucleotides in a 5' to 3' direction as it slides toward the opening of the replication fork
  - **Lagging strand**
    - Synthesis is also in the 5' to 3' direction
      - However it occurs away from the replication fork
    - Many RNA primers are required
    - DNA pol III uses the RNA primers to synthesize small DNA fragments (1000 to 2000 nucleotides each)
      - These are termed **Okazaki fragments** after their discoverers



# Bacterial DNA Polymerase

- DNA Polymerase I and II involved in repair and polymerization of part of one strand
- DNA Polymerase III is major component of replisome
- Multi-subunit, **asymmetric** dimer
- Many activities



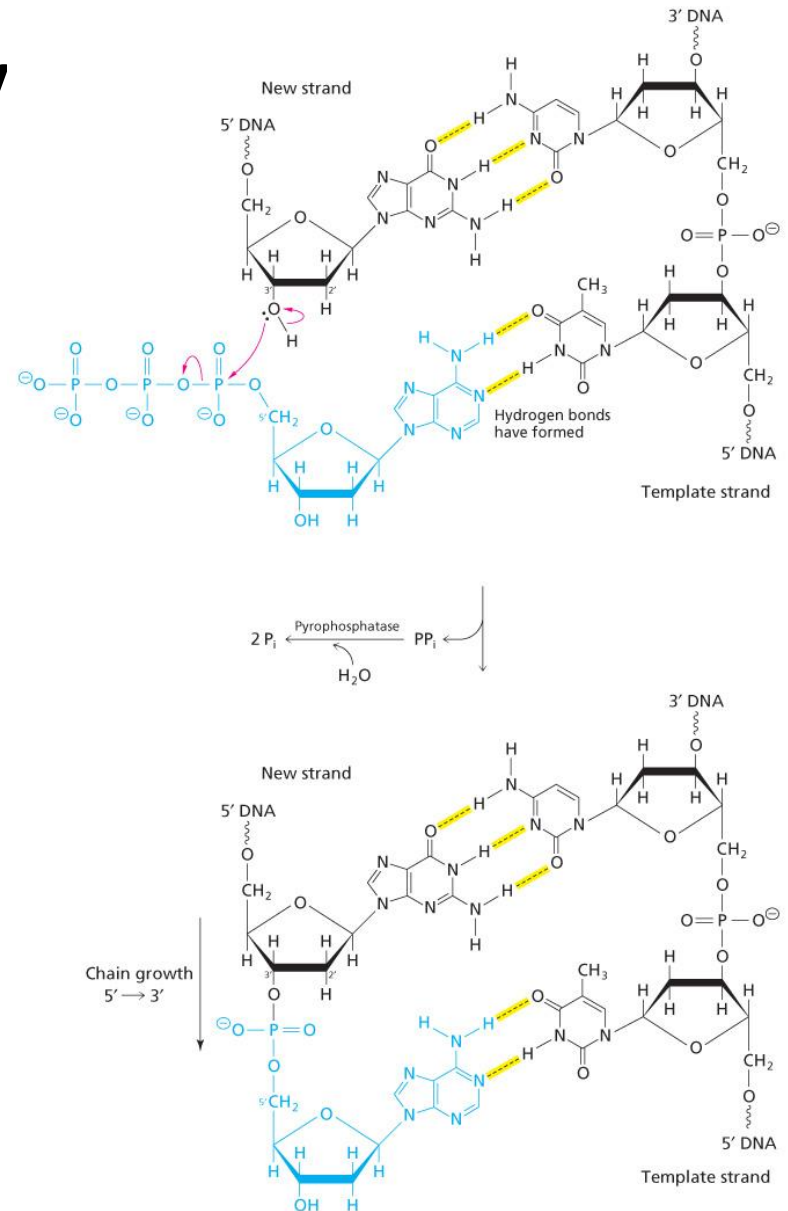
# Mechanism of Action of DNA Polymerases

# The Reaction of DNA Polymerase

- DNA polymerases catalyzes a phosphodiester bond between the
  - Innermost phosphate group of the incoming deoxynucleoside triphosphate
    - AND
  - 3'-OH of the sugar of the previous deoxynucleotide
- In the process, the last two phosphates of the incoming nucleotide are released
  - In the form of pyrophosphate ( $PP_i$ )

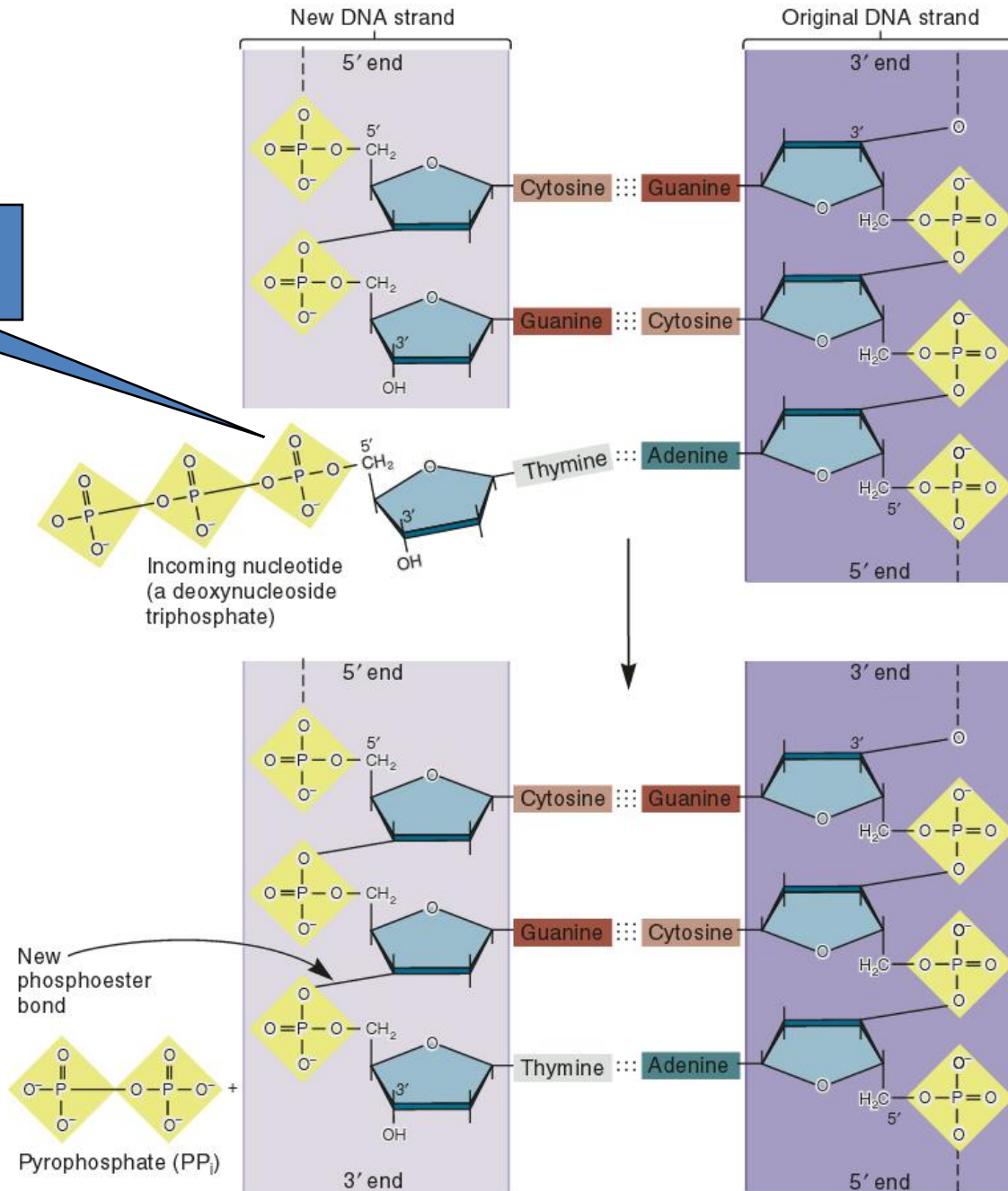
# Polymerase Activity

- dNTP is substrate
- Added to 3' end of growing chain
  - PRIMER is required
- 5' → 3' growth
- Selectivity based on H-bonding
  - TEMPLATE is required
- Pyrophosphate generated



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Innermost phosphate



# DNA Polymerases III



## Points to be remembered.....

1. A **core enzyme** consists of the subunits of an enzyme that are needed for catalytic activity.
2. Some enzymes require non-protein molecules called **cofactors** to be bound for activity.
3. Cofactors can be either **inorganic** (e.g., metal ions and iron-sulfur clusters) or **organic compounds** (e.g., flavin and heme).
4. Organic cofactors can be either
  - a. **Coenzymes**, which are released from the enzyme's active site during the reaction
  - b. **Prosthetic groups**, which are tightly bound to an enzyme.
5. Organic prosthetic groups can be covalently bound (e.g., biotin in enzymes such as pyruvate carboxylase).

# Points to be remembered.....

Enzymes that require a cofactor but do not have one bound are called **apoenzymes** or **apoproteins**.

An enzyme together with the cofactor(s) required for activity is called a **holoenzyme** / **haloenzyme**.

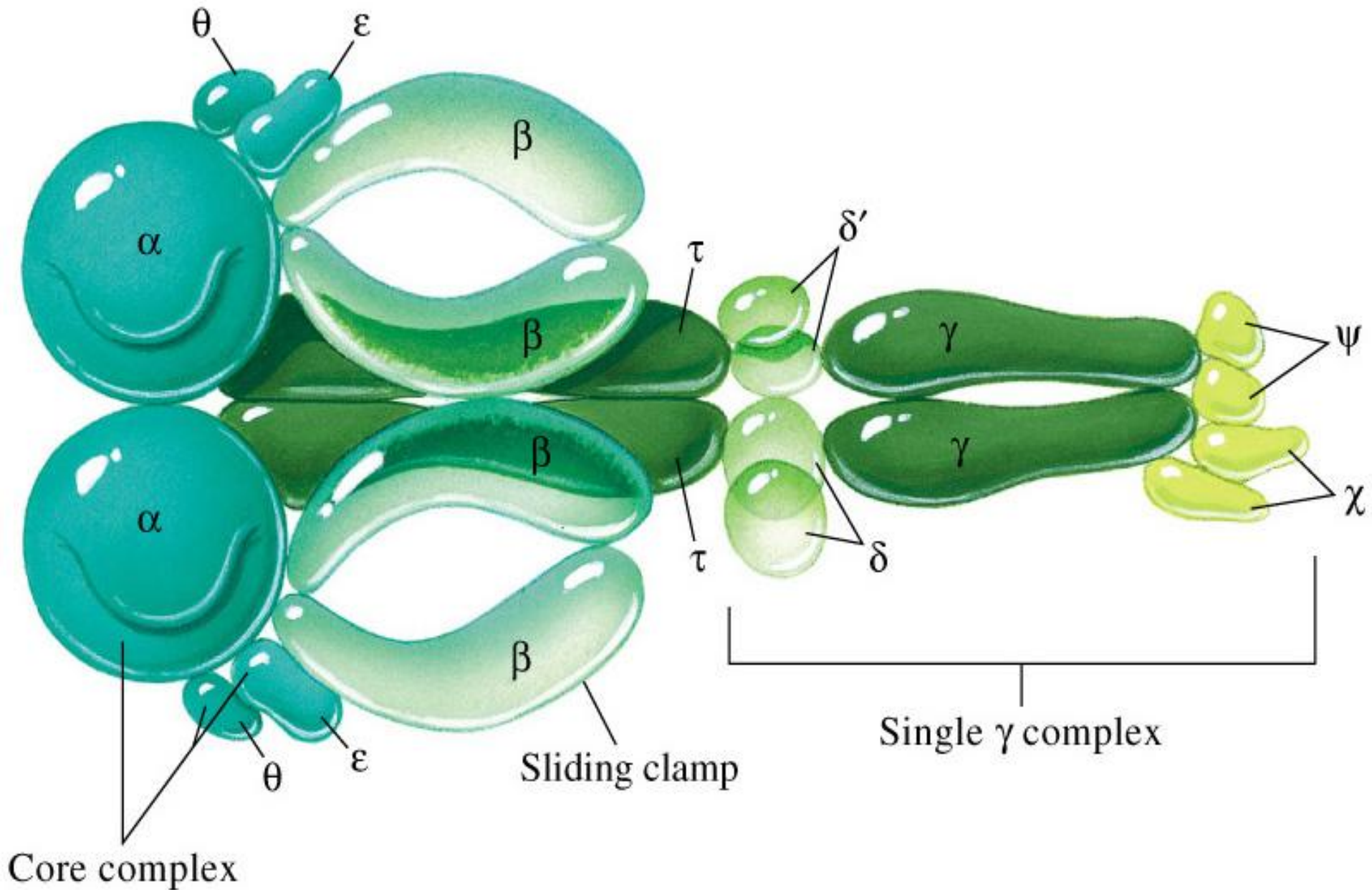
The term **holoenzyme** can also be applied to enzymes that contain **multiple protein subunits**, such as the **DNA polymerases**.

Here the **holoenzyme** is the complete complex containing all the subunits needed for activity.

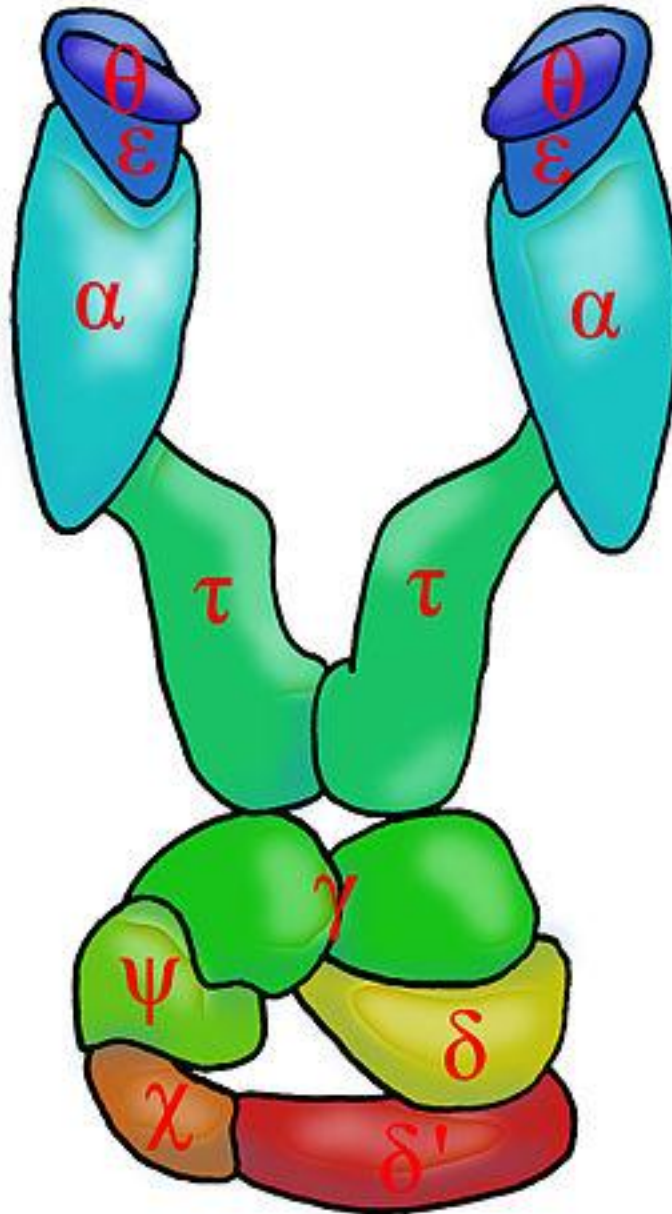
# DNA Polymerase III Holoenzyme

- The holoenzyme is a complex of ~10 proteins of a total mass of ~900 kD.
- The holoenzyme is organized into four types of subcomplex:
  - Two copies of catalytic core, each core contains:
    - ❖ One copy of  $\alpha$ -subunit (5' → 3' Polymerase activity)
    - ❖ One copy of  $\epsilon$ -subunit (3' → 5' Proofreading exonuclease activity)
    - ❖ One copy of  $\theta$ -subunit (stimulate exonuclease activity)
    - ❖ Two copies of dimerizing subunit  $\tau$ , which link the two catalytic core
  - Two copies of  $\beta$ -clamp, responsible for holding catalytic cores on their template. Each clamp consists of a homodimer of  $\beta$  subunits, the  $\beta$  ring → ensures processivity
  - One copy of clamp loader →  $\gamma$  complex: a group of five proteins, places clamp on DNA ( $\gamma\delta\delta'\chi\psi$ )

# DNA Polymerase III Holoenzyme (Replicase)



**Schematic picture of *DNA polymerase III*\* (with subunits).**



**MW ~900 kd**

# SUMMARY

**DNAPIII holoenzyme is made up of FOUR types of sub-complexes:**

## Sub-complex 1

**2 DNA Pol III enzymes**, each comprising  $\alpha$ ,  $\epsilon$  and  $\theta$  subunits.

- the  $\alpha$  subunit (encoded by the *dnaE* gene) has the polymerase activity.
- the  $\epsilon$  subunit (*dnaQ*) has 3'→5' exonuclease activity.
- the  $\theta$  subunit (*holE*) stimulates the  $\epsilon$  subunit's proofreading.

## Sub-complex 2

**2  $\beta$  units** (*dnaN*) which act as sliding DNA clamps, they keep the polymerase bound to the DNA.

## Sub-complex 3

**2  $\tau$  units** (*dnaX*) which act to dimerize two of the core enzymes ( $\alpha$ ,  $\epsilon$ , and  $\theta$  subunits).

## Sub-complex 4

**1  $\gamma$  unit** (also *dnaX*) which acts as a clamp loader for the lagging strand Okazaki fragments, helping the two  $\beta$  subunits to form a unit and bind to DNA. The  $\gamma$  unit is made up of 5  $\gamma$  subunits which include 2  $\gamma$  subunits, 1  $\delta$  subunit (*holA*), and 1  $\delta'$  subunit (*holB*). The  $\delta$  is involved in copying of the lagging strand.

**$\chi$  (*holC*) and  $\Psi$  (*holD*)** which form a 1:1 complex and bind to  $\gamma$  or  $\tau$ .  $\chi$  can also mediate the switch from RNA primer to DNA.

## TABLE 11.2

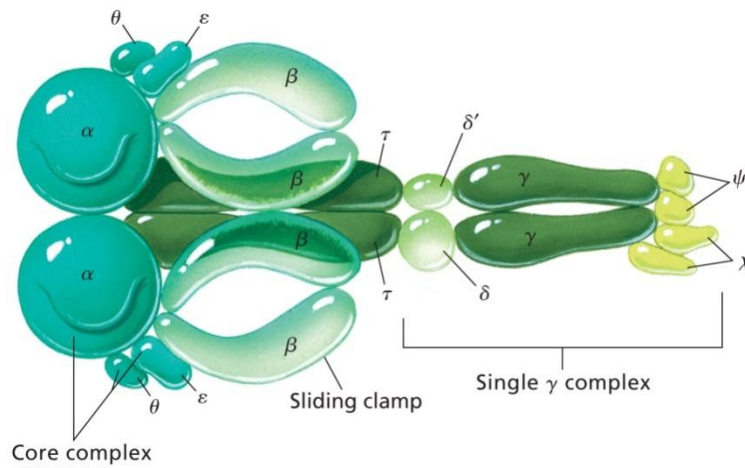
### Subunit Composition of DNA Polymerase (polIII) Holoenzyme from *E. coli*

Subunit	Function
$\alpha$	Synthesizes DNA
$\epsilon$	3' to 5' proofreading (removes mismatched nucleotides)
$\theta$	Accessory protein that stimulates the proofreading function
$\tau$	Promotes the dimerization of two polIII proteins together at the replication fork; also, stimulates DNA helicase
$\beta$	Clamp protein, which allows DNA polymerase to slide along the DNA without falling off
$\gamma$	Clamp loader protein; initially helps the clamp protein to bind to the DNA
$\delta$	Accessory protein that binds to $\beta$
$\delta'$	Accessory protein that stimulates $\gamma$
$\psi$	Accessory protein that stimulates $\gamma$
$\chi$	Accessory protein that binds to single-strand binding protein

**Table 20.1 Subunits of DNA polymerase III holoenzyme**

Subunit	$M_r$	Gene	Activity
$\alpha$	130 000	<i>polC/dnaE</i>	Polymerase
$\epsilon$	27 000	<i>dnaQ/mutD</i>	3' $\rightarrow$ 5' exonuclease
$\theta$	8846	<i>holE</i>	?
$\beta$	40 000	<i>dnaN</i>	Forms sliding clamp
$\tau$	71 000	<i>dnaX</i>	Enhances dimerization of core; ATPase
$\gamma$	47 000	<i>dnaX</i>	Enhance processivity; assist in replisome assembly
$\delta$	38 700	<i>holA</i>	
$\delta'$	36 900	<i>holB</i>	
$\chi$	16 600	<i>holC</i>	
$\psi$	15 174	<i>holD</i>	

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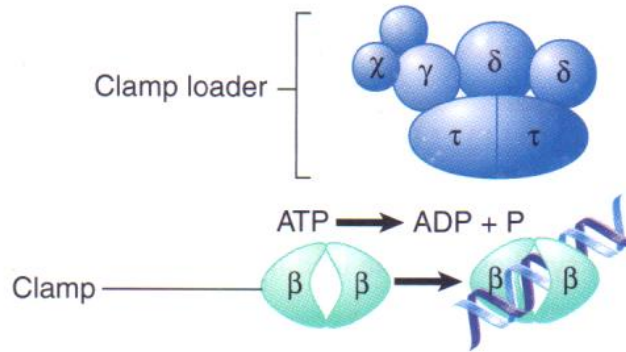
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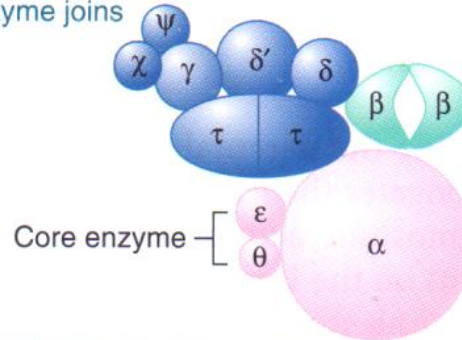
# Activities of DNA Pol III

- ~900 kd
- Synthesizes **both** leading and lagging strand
- Can only extend from a primer (either RNA or DNA), cannot initiate synthesis
- 5' → 3' polymerizing activity
- 3' → 5' exonuclease activity
- **Very Weak** 5' → 3' exonuclease activity  
(Biological function of this property not clear)

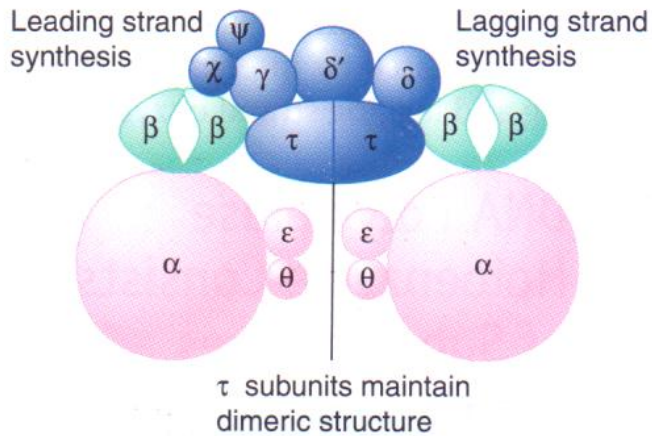
Clamp loader cleaves ATP to load clamp on DNA



Core enzyme joins



tau + second core joins to give a symmetric dimer



# DNA Polymerase III is a Processive Enzyme

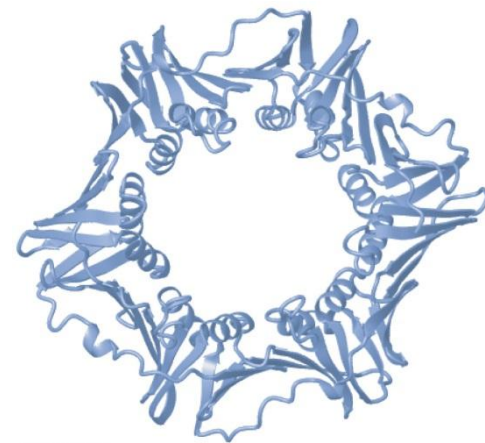
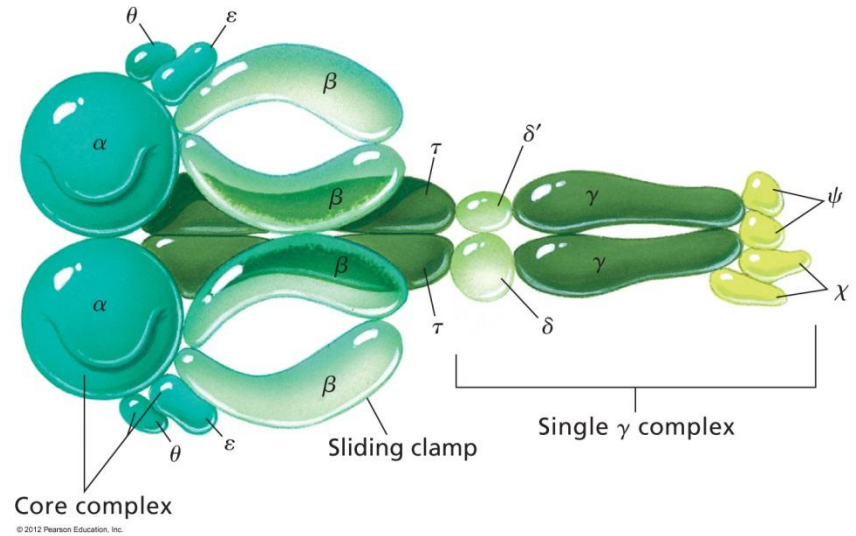
- DNA polymerase III remains attached to the template as it is synthesizing the daughter strand
- This **processive** feature is due to several different subunits in the DNA pol III holoenzyme
  - $\beta$  subunit is in the shape of a ring
    - It is termed the clamp protein
  - $\gamma$  subunit is needed for  $\beta$  to initially clamp onto the DNA
    - It is termed the clamp-loader protein
  - $\delta$ ,  $\delta'$  and  $\chi$  subunits are needed for the optimal function of the  $\alpha$  and  $\epsilon$  subunits

# DNA Polymerase III is a Processive Enzyme

- The effect of processivity is quite remarkable
  - In the absence of the  $\beta$  subunit
    - DNA pol III falls off the DNA template after a few dozen nucleotides have been polymerized
    - Its rate is ~ 20 nucleotides per second
  - In the presence of the  $\beta$  subunit
    - DNA pol III stays on the DNA template long enough to polymerize up to 50,000 nucleotides
    - Its rate is ~ 750 nucleotides per second

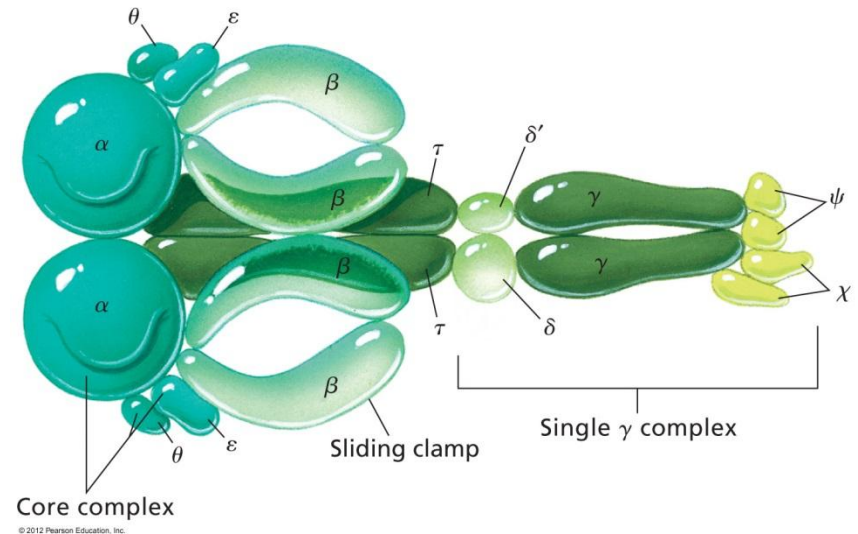
# Processivity

- Replisome stays attached to replication fork
- Beta subunit is a sliding clamp
- Higher efficiency
- Few replisomes needed



# Polymerase III Proofreads

- Incorrect base pair every  $10^5$  bp
- Epsilon subunit is proofreader
- Unmatched base pairs have different conformation
- $3' \rightarrow 5'$  exonuclease activity
- 99% of errors corrected here
- 99% of remaining errors are repaired



**TABLE 5–1** The Three Steps That Give Rise to High-Fidelity DNA Synthesis

REPLICATION STEP	ERRORS PER NUCLEOTIDE POLYMERIZED
5'→3' polymerization	$1 \times 10^5$
3'→5' exonucleolytic proofreading	$1 \times 10^2$
Strand-directed mismatch repair	$1 \times 10^2$
Total	$1 \times 10^9$

## Genome Size

***E. coli* K12**     **$4.6 \times 10^6$  bp**

(One error per genome per 1000 bacterial replication cycle)

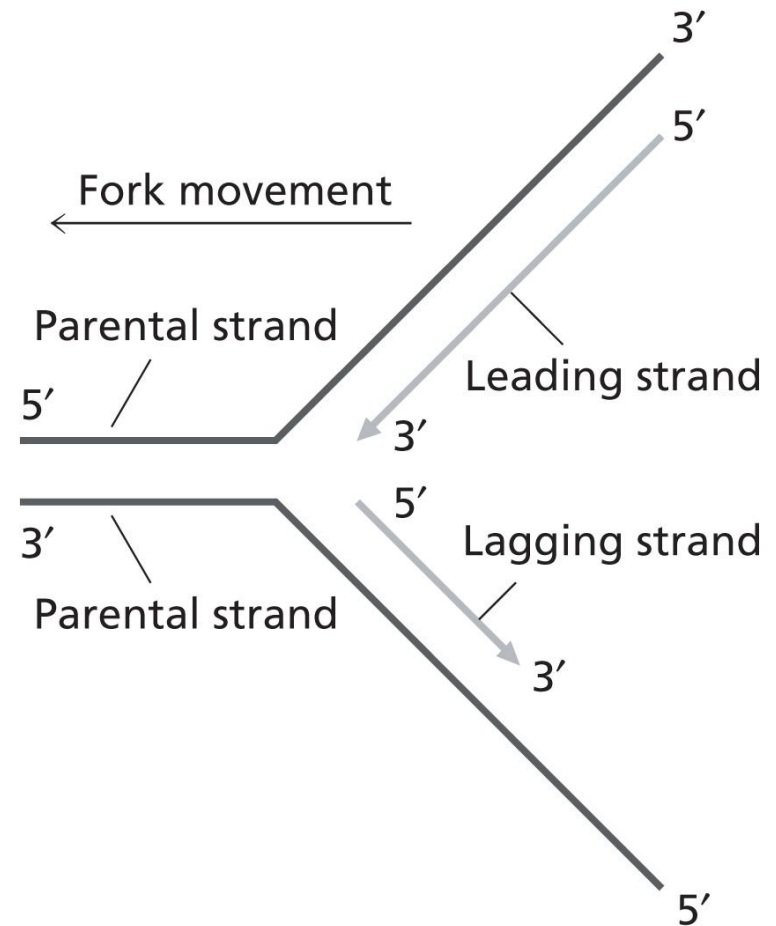
**Mouse**             **$2.8 \times 10^9$  bp**

**Human**             **$3.3 \times 10^9$  bp**

(~3 error per replication cycle)

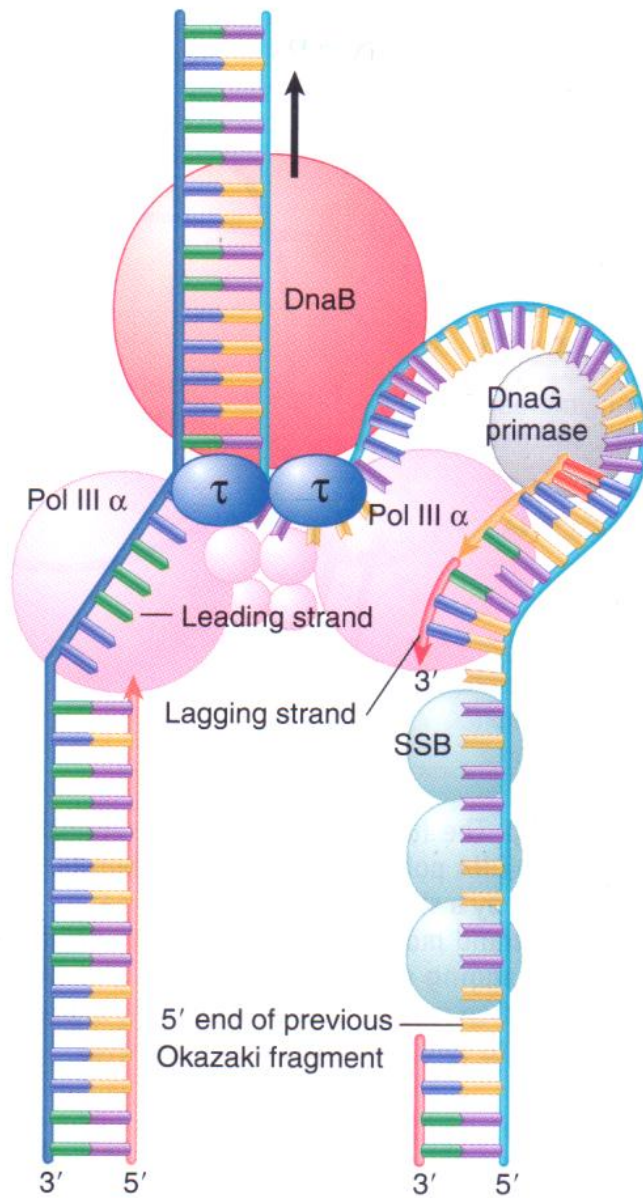
# Polymerase Synthesizes Both Strands Simultaneously!

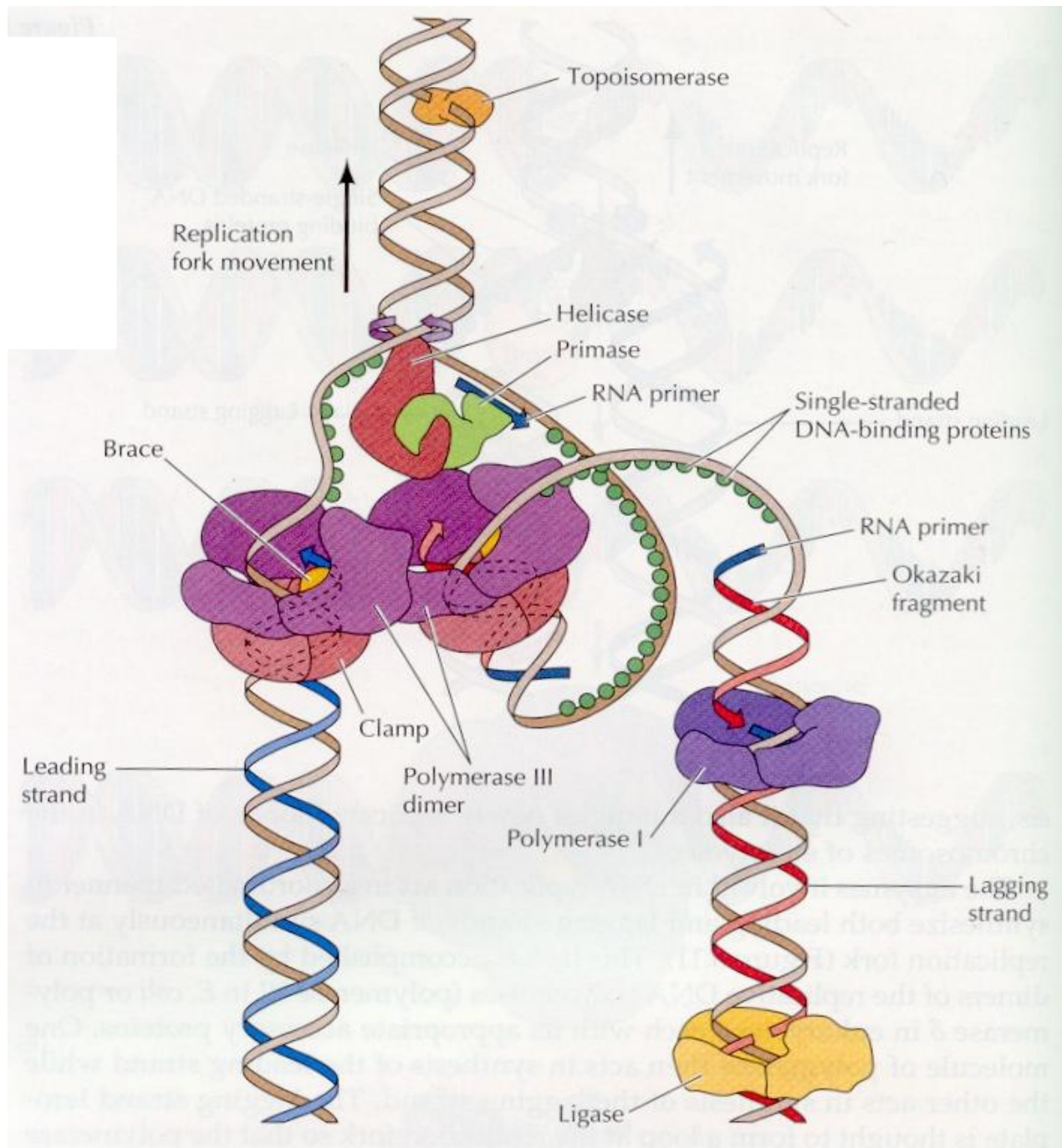
- Synthesis is  $5' \rightarrow 3'$
- Leading Strand built on the  $3' \rightarrow 5'$  template
- Lagging Strand built on the  $5' \rightarrow 3'$  template
  - Discontinuous
  - Okazaki fragments



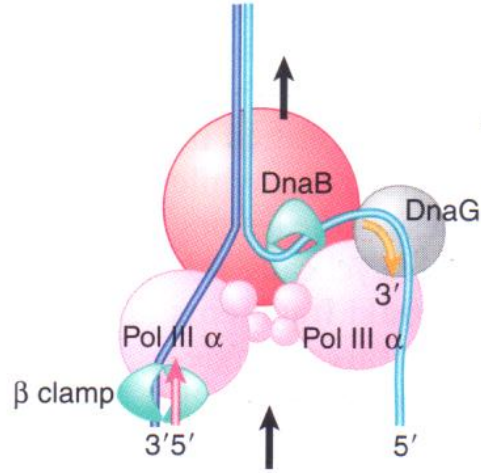
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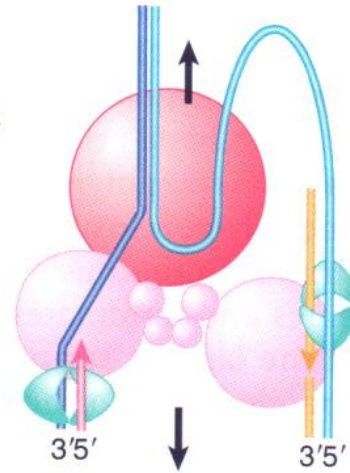




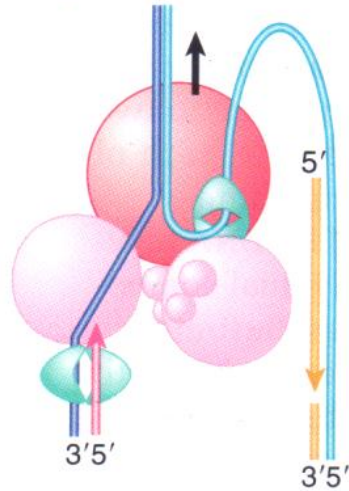
1. Initiation of Okazaki fragment



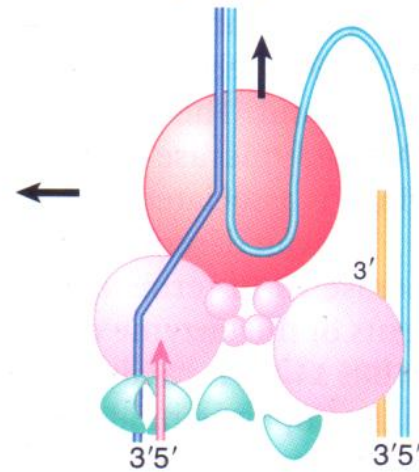
2. Termination of Okazaki fragment



4.  $\beta$  clamp associates

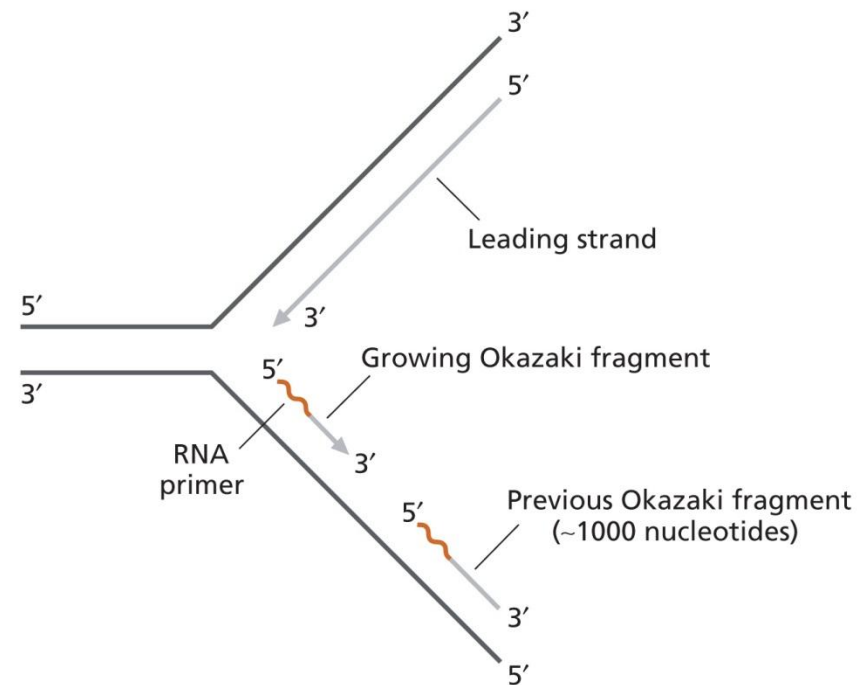


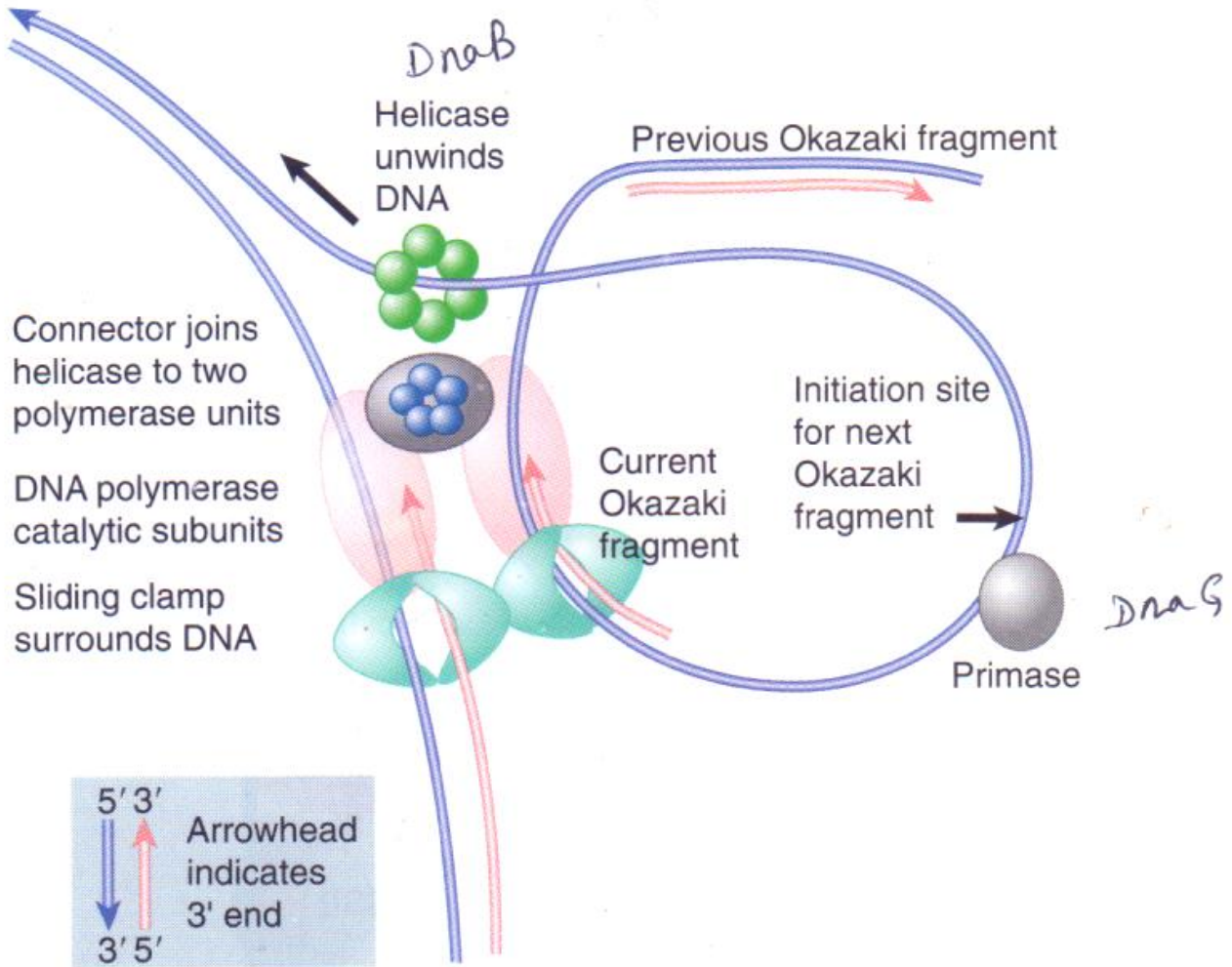
3.  $\beta$  clamp dissociates



# Primase

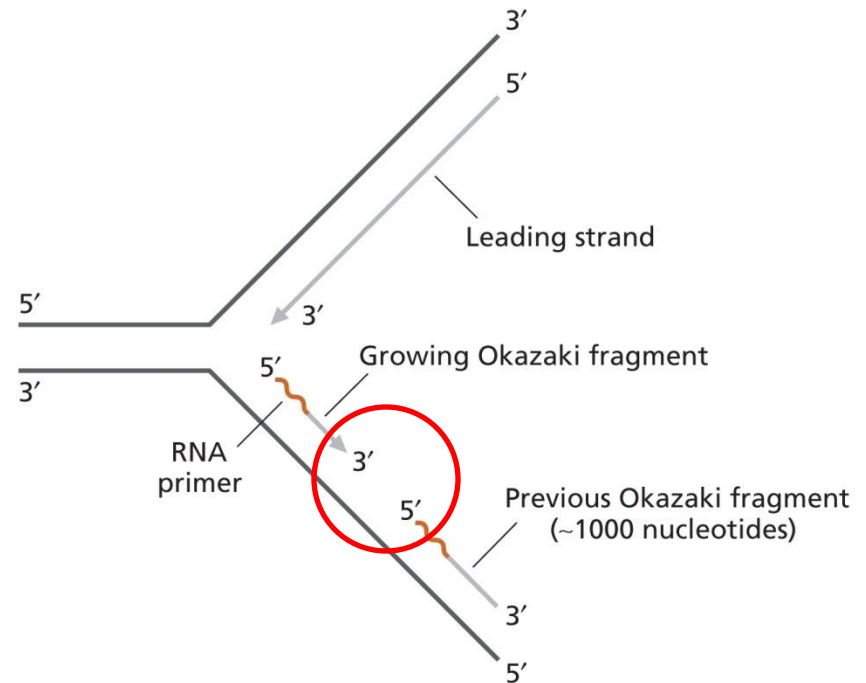
- RNA primer is needed for each polymerization
- RNA primer is made by primase enzyme
  - Part of the primosome complex
- Primosome, along with DNA Polymerase III, is part of replisome





# Closing the Nick

- Three actions needed
- Removal of RNA primer
  - DNA polymerase I
- Filling in the nick with DNA
  - DNA polymerase I
- Forming bond between adjacent fragments
  - DNA ligase



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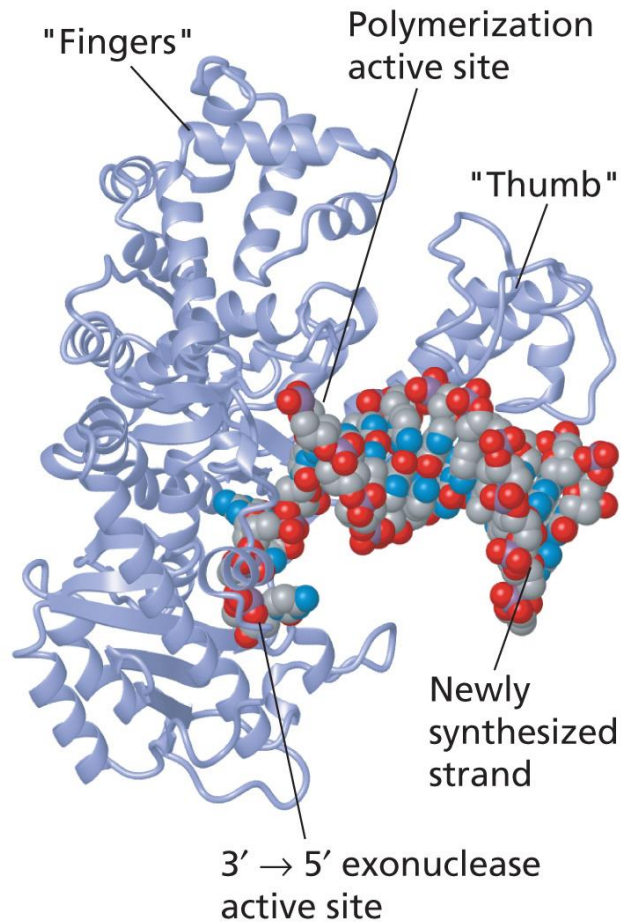
# DNA Polymerases I

# DNA Polymerase I

- Two activities similar to DNA polymerase III
  - 5' → 3' polymerase
    - Extend chain
  - 3' → 5' exonuclease
    - Proofreading
- Unique activity
  - 5' → 3' exonuclease
    - Removes RNA primer



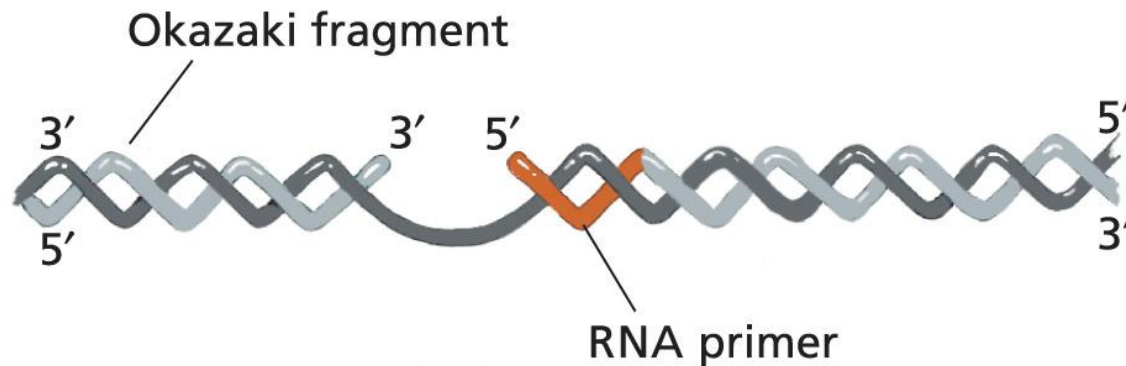
# Klenow Fragment



- DNA polymerase I was first discovered
  - Kornberg Nobel prize
- Can be cut into two pieces that retain activity
- Klenow fragment
  - Bigger piece with 5' → 3' exonuclease removed
  - “Usable” form of DNA polymerase III

# Three Step Process

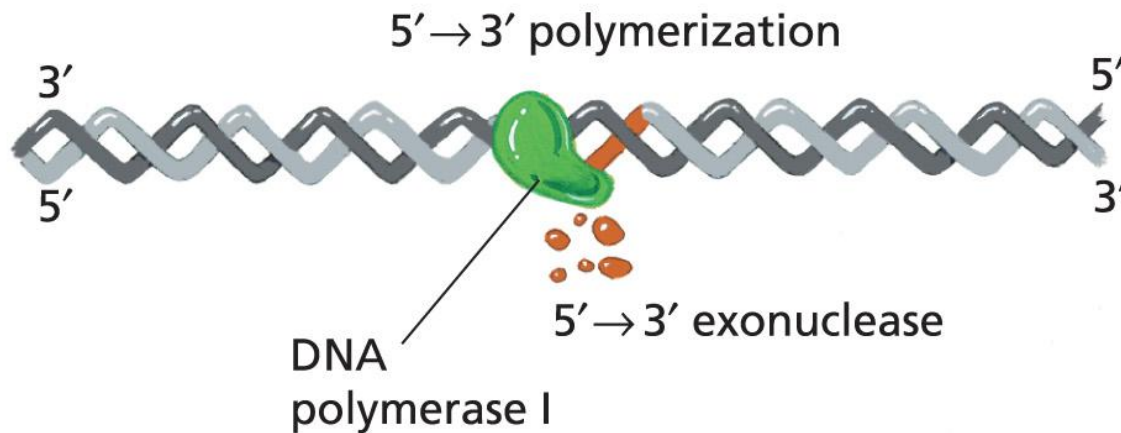
- (a) Completion of Okazaki fragment synthesis leaves a nick between the Okazaki fragment and the preceding RNA primer on the lagging strand.



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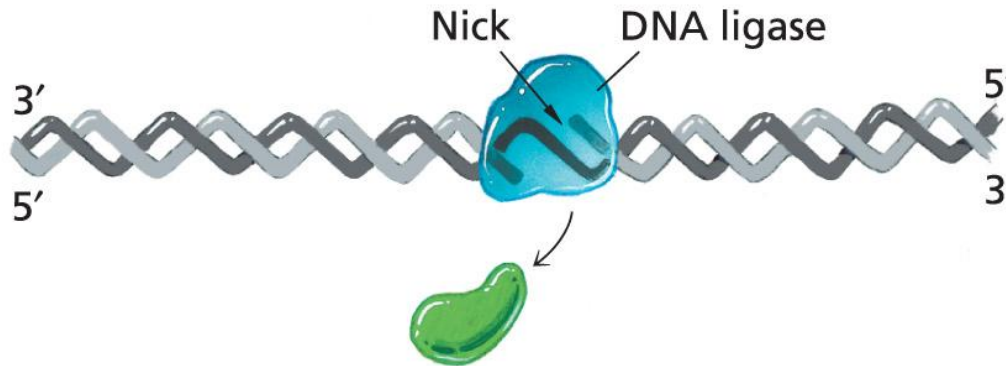
# Step 1

- (b) DNA polymerase I extends the Okazaki fragment while its 5' → 3' exonuclease activity removes the RNA primer. This process, called nick translation, results in movement of the nick along the lagging strand.



# Step 2

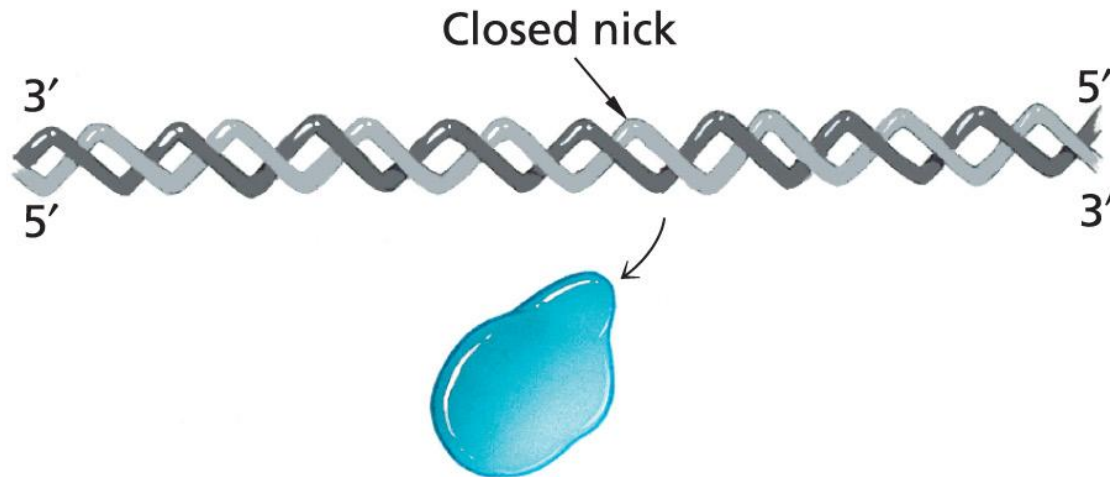
- (c) DNA polymerase I dissociates after extending the Okazaki fragment 10–12 nucleotides. DNA ligase binds to the nick.



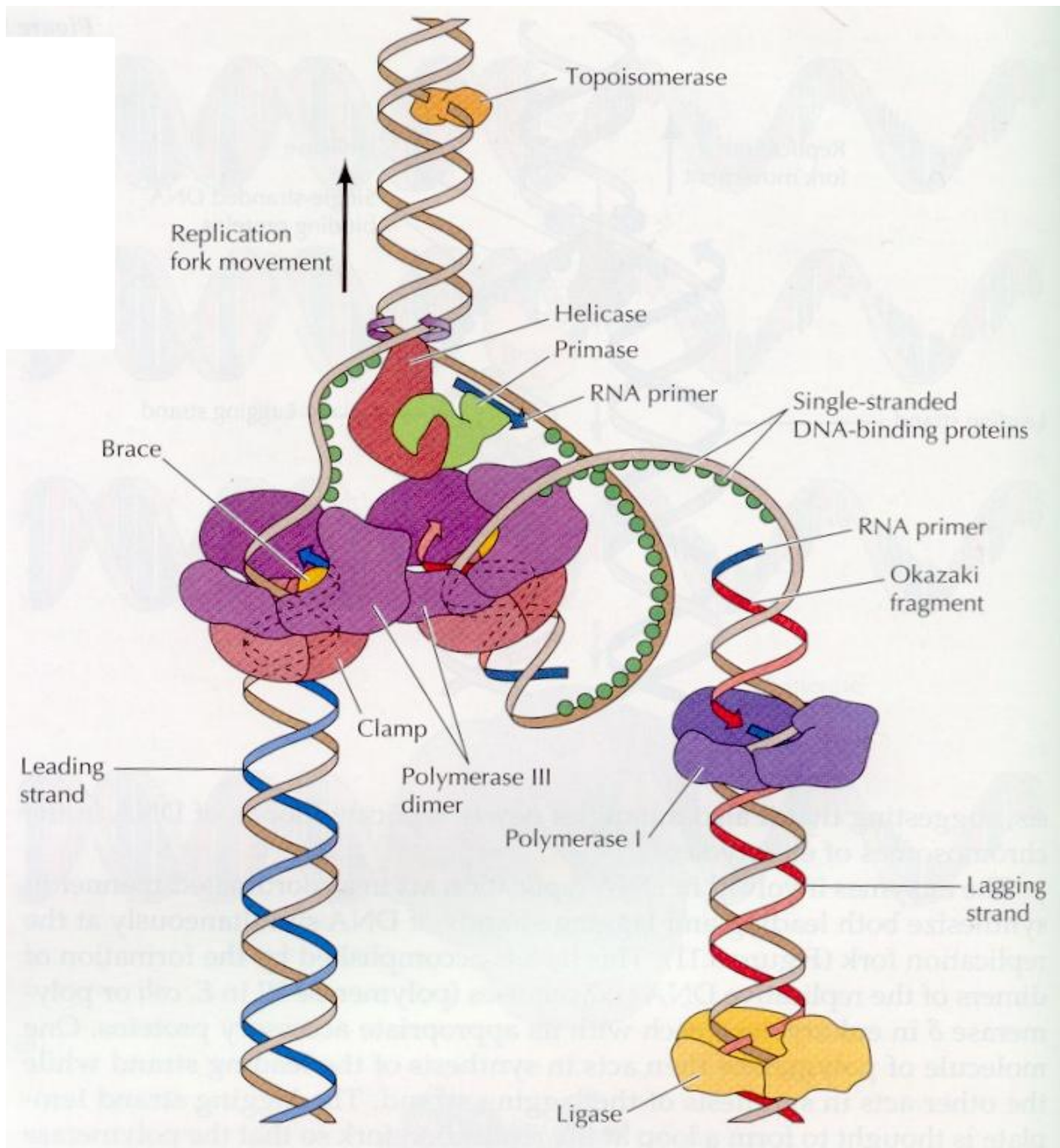
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# Step 3

- (d) DNA ligase catalyzes formation of a phosphodiester linkage, which seals the nick, creating a continuous lagging strand. The enzyme then dissociates from the DNA.



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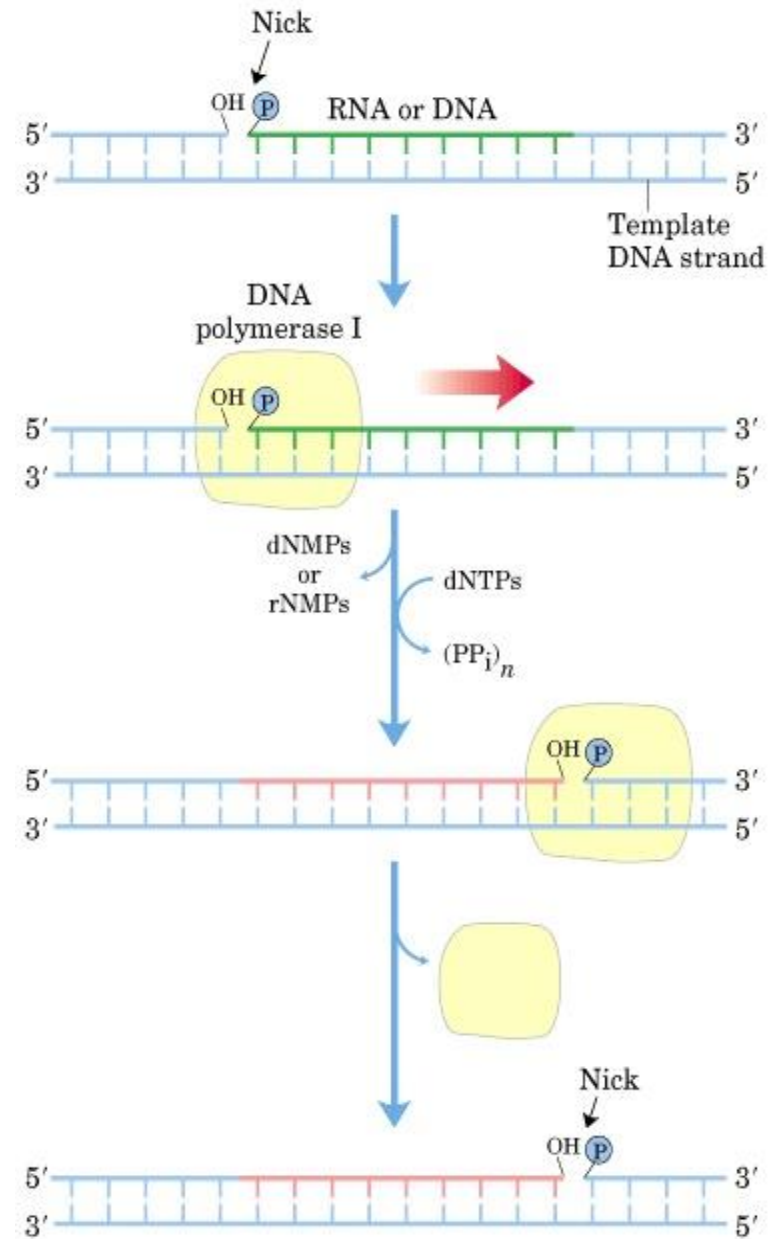
# Nick Translation Reaction

# Nick Translation

Requires 5'-3' activity of DNA pol I

## Steps

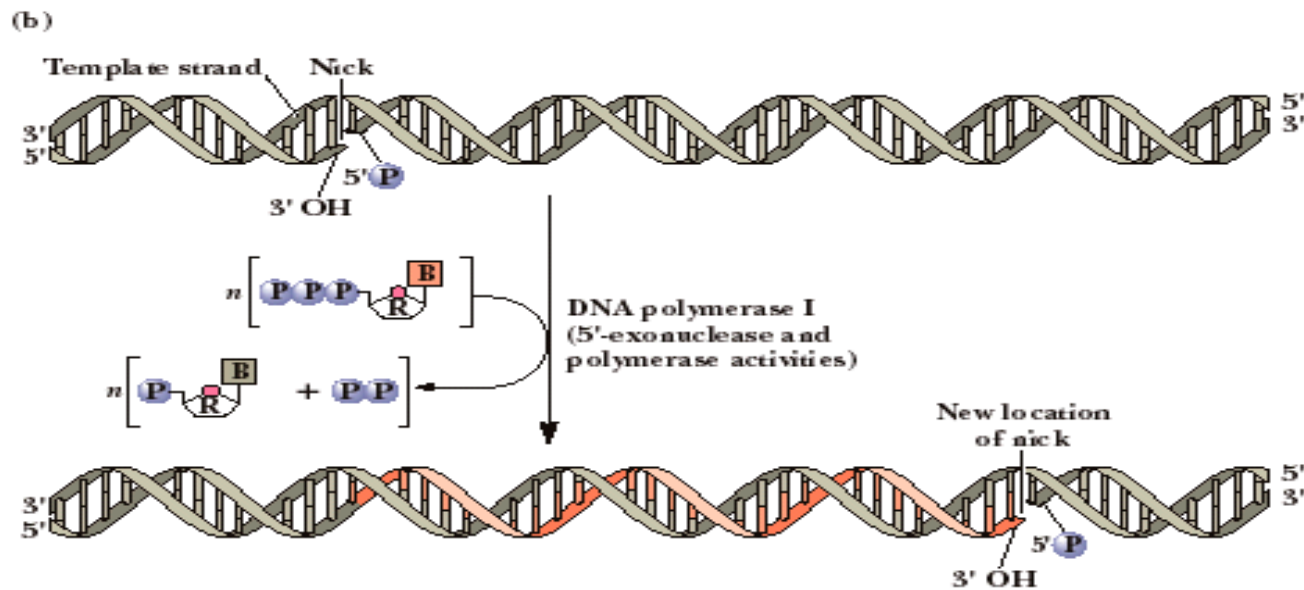
1. At a nick (free 3' OH) in the DNA the DNA pol I binds and digests nucleotides in a 5'-3' direction
2. The DNA polymerase activity synthesizes a new DNA strand
3. A nick remains as the DNA pol I dissociates from the ds DNA.
4. The nick is closed via DNA ligase





# Nick Translation 2

5'-exonuclease activity, working together with the polymerase, accomplishes "nick translation"



Saunders Colleg

**This activity is critical in primer removal**

# SUMMARY

**TABLE 11.1**

**Proteins Involved in *E. coli* DNA Replication**

<b>Common Name</b>	<b>Function</b>
DnaA protein	Binds to DnaA boxes within the origin to initiate DNA replication
DNA helicase (DnaB)	Separates double-stranded DNA
DnaC protein	Aids DnaA in the recruitment of helicase to the origin
Topoisomerase	Removes supercoils ahead of the replication fork
DNA primase	Synthesizes short RNA primers
DNA polymerase (polIII)	Synthesizes DNA in the leading and lagging strands
DNA polymerase (polI)	Removes RNA primers, fills in gaps
DNA ligase	Covalently attaches adjacent Okazaki fragments
Tus	Binds to <i>ter</i> sequences and prevents the advancement of the replication fork