B.Sc (Hons) Microbiology (CBCS Structure)

C-7: Molecular Biology

Unit 2: Replication of DNA

DNA Replication Enzymes and M

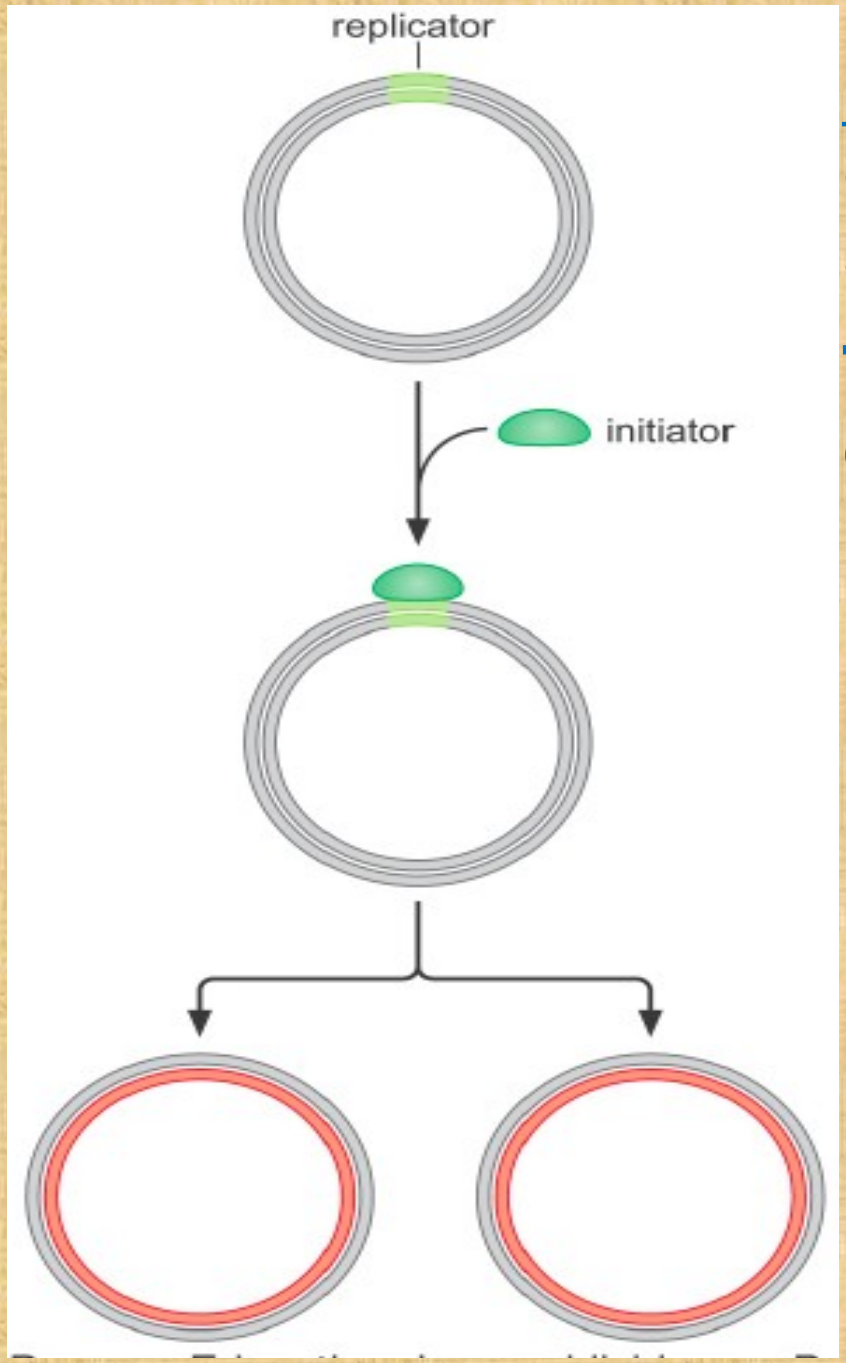
Dr. Rakesh Kumar Gupta
Department of Microbiology
Ram Lal Anand College
New Delhi - 110021

Chapter 8: Molecular Biology of the Gene by Watson *et.*

Specific genomic DNA sequences direct the initiation of DNA replication

Origins of replication, the sites at which DNA unwinding and initiation of replication occur.

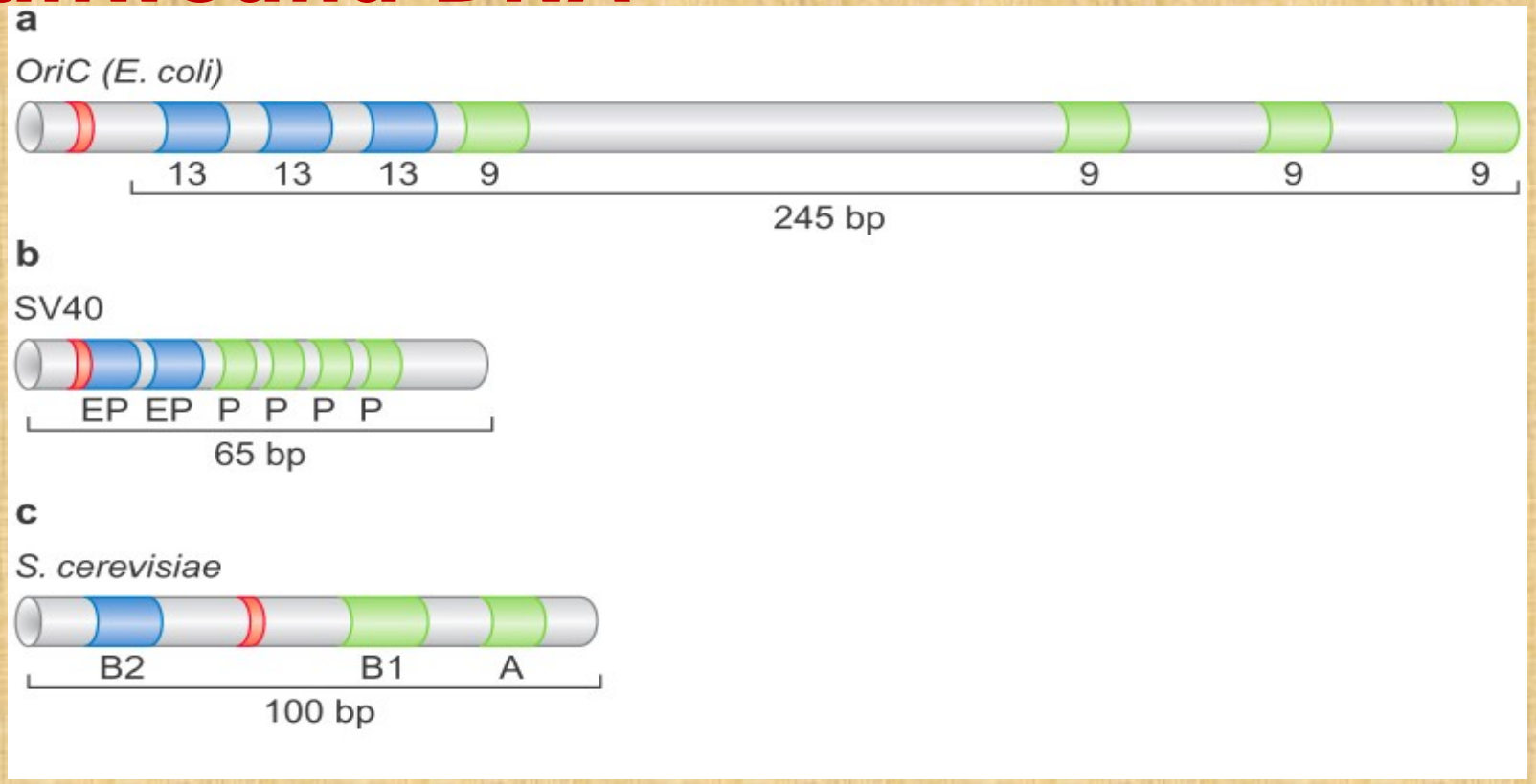
- Proposed by Jacob and Brenner in 1963
- All the DNA replicated from a particular origin is a **replicon**
- Two components, **replicator** and **initiator**, control the initiation of replication



Replicator: the entire site of cis-acting DNA sequences sufficient to direct the initiation of DNA replication

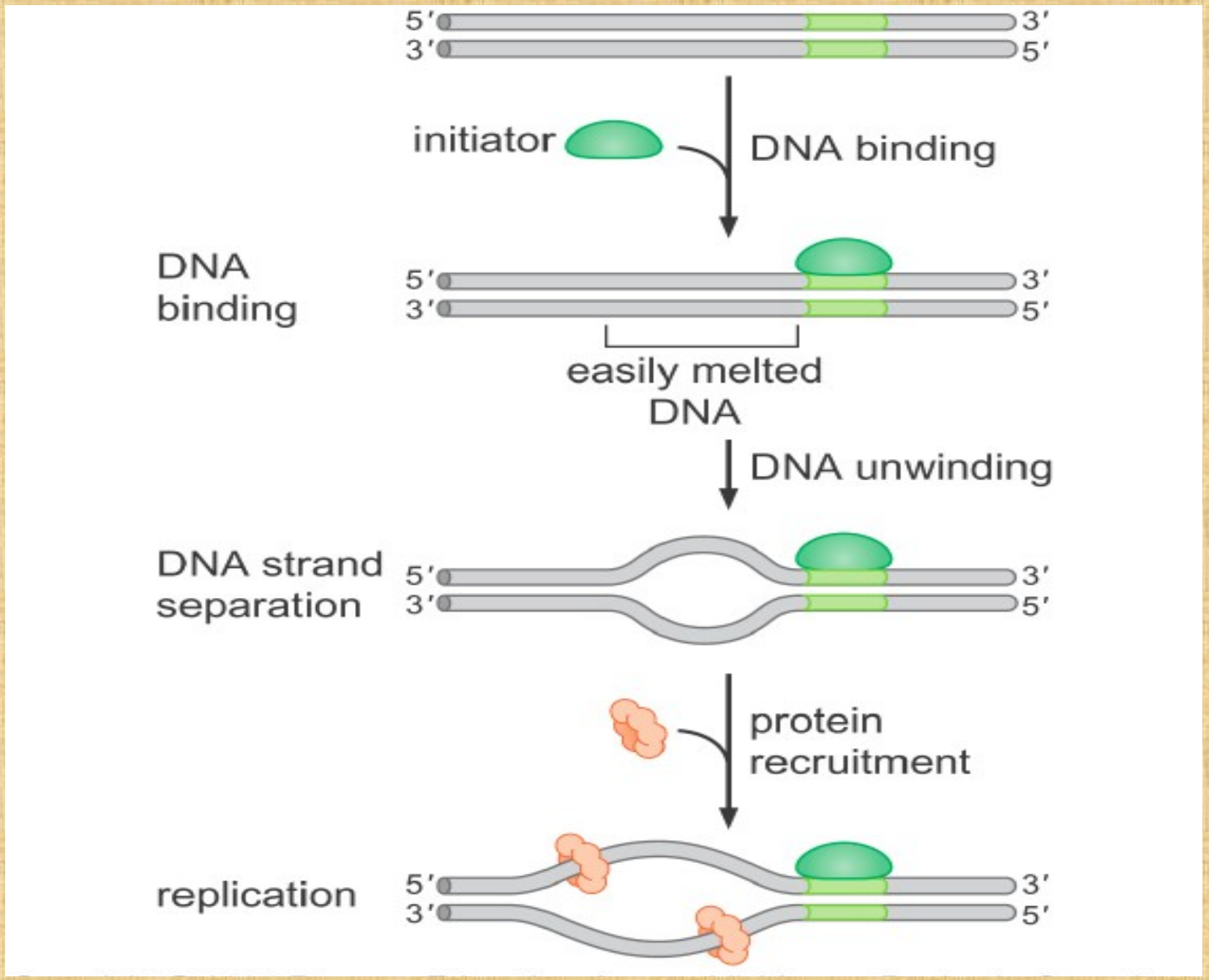
Initiator protein: specifically recognizes a DNA element in the replicator and activates the initiation of replication

Replicator sequences include initiator binding sites and easily unwound DNA



Binding and Unwinding: origin selection and activation by the initiator protein

- **Three different functions of initiator protein: (1) binds to replicator, (2) distorts/unwinds a region of DNA, (3) interacts with and recruits additional replication factors**
- **DnaA in *E. coli* *(all 3 functions)*, origin recognition complex (ORC) in eukaryotes *(functions 1 & 3)***


5′
3′
3′
5′
initiator
DNA binding
DNA
binding
5′
3′
3′
5′
easily melted
DNA
DNA unwinding
DNA strand
separation
5′
3′
3′
5′
protein
recruitment
replication
5′
3′
3′
5′

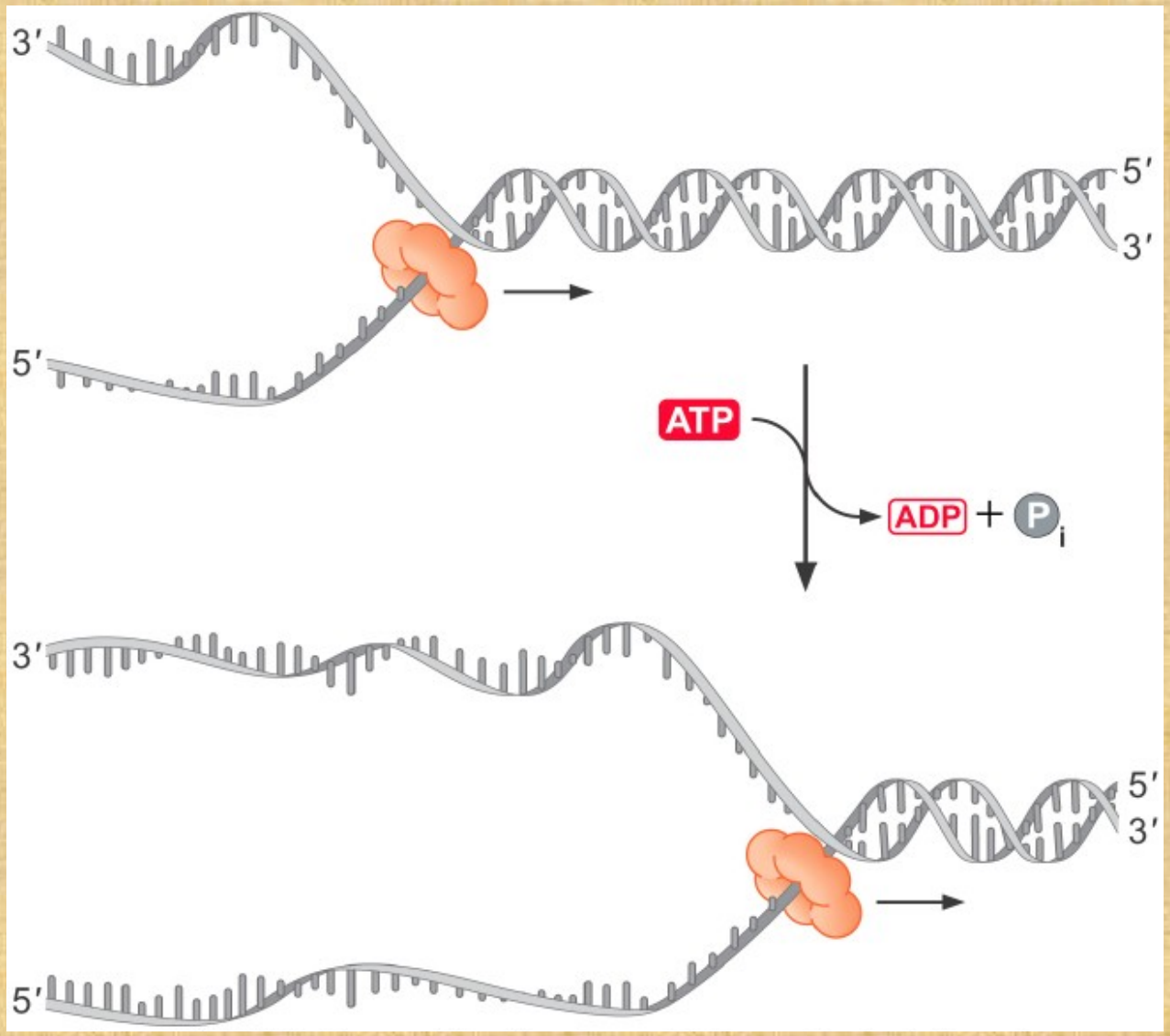
Protein-protein and protein-DNA interactions direct the initiation process

- Initiating replication in bacteria
- DnaA recruits the DNA helicase DnaB and the helicase loader DnaC
- DnaB interacts with primase to initiate RNA primer synthesis.

DNA helicases unwind the double helix in advance of the replication fork

E. coli – DnaB, hexamer, 12 types, 5’-3’ polarity, single strand DNA affinity

S. cerevisiae and human– Mcm complex

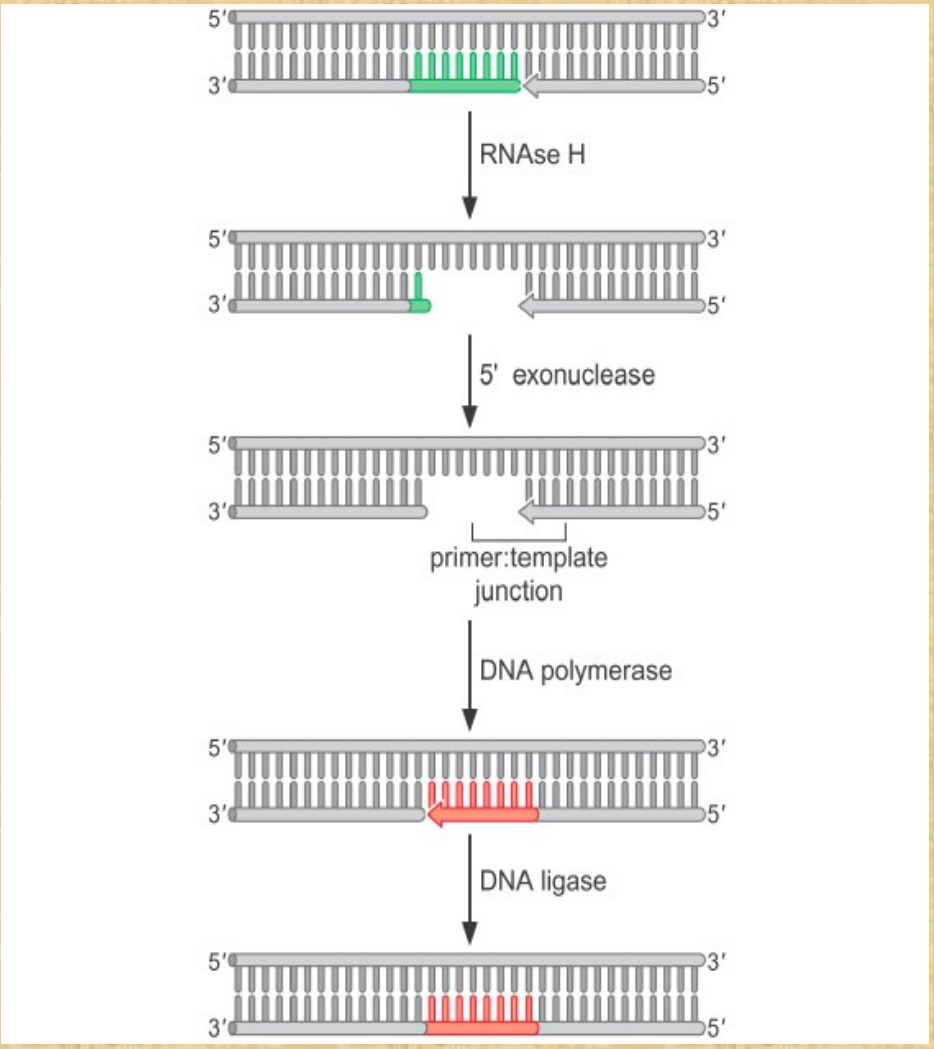


The initiation of a new strand of DNA require an RNA primer

- **Primase is a specialized RNA polymerase dedicated to making short RNA primers on an ssDNA template. Do not require specific DNA sequence for binding.**
- **A dnaG gene product, 60kD, single polypeptide product, 4-15 bases RNA primer in *E. coli.***
- **Primase (PRI1/PRI2) in *S. cerevisiae* and Primase in human**
- **DNA Pol can extend both RNA and DNA primers annealed to DNA template**

RNA primers must be removed to complete DNA replication

A joint efforts of RNase H, DNA polymerase & DNA ligase

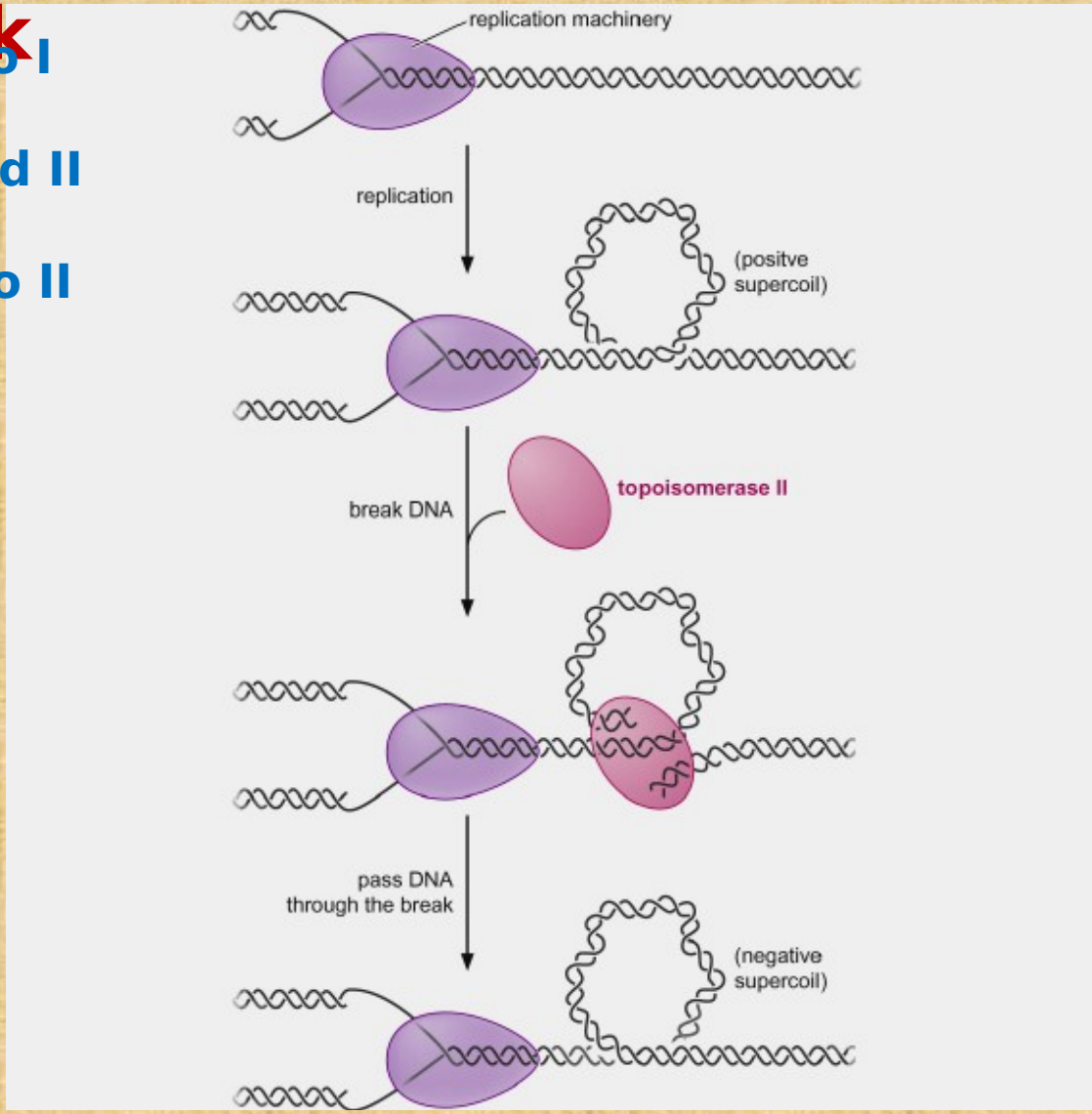


Topoisomerase removes supercoils produced by DNA unwinding at the replication fork

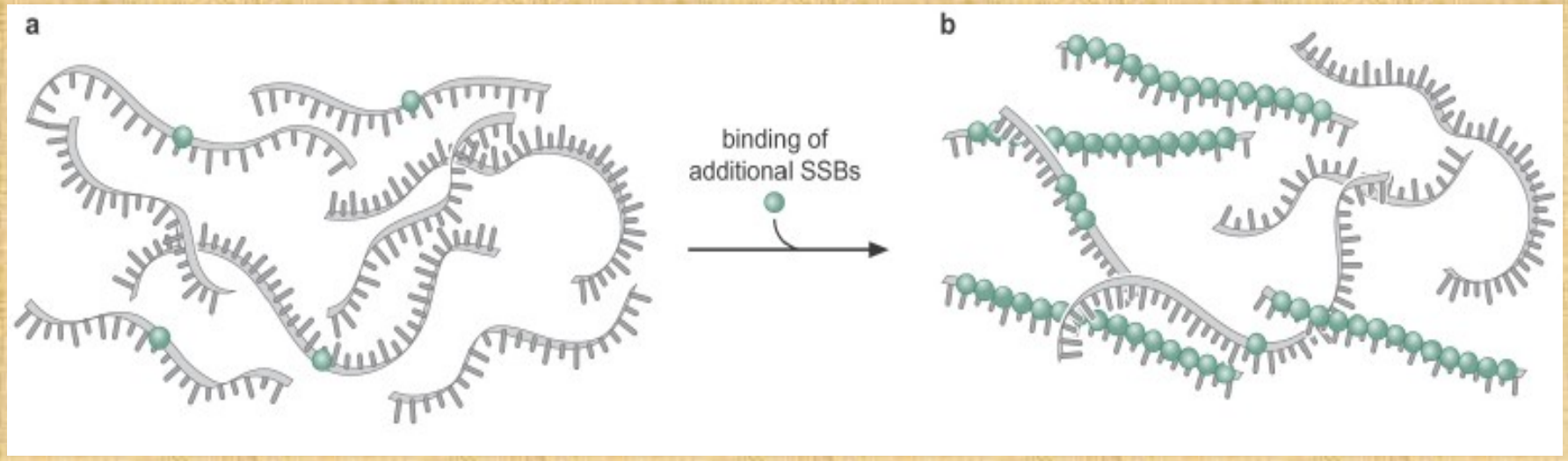
E. coli **– Gyrase and Topo I**

S. cerevisiae **– Topo I and II**

Human – Topo I and Topo II



Single-stranded binding proteins (SSBs) stabilize single-stranded DNA

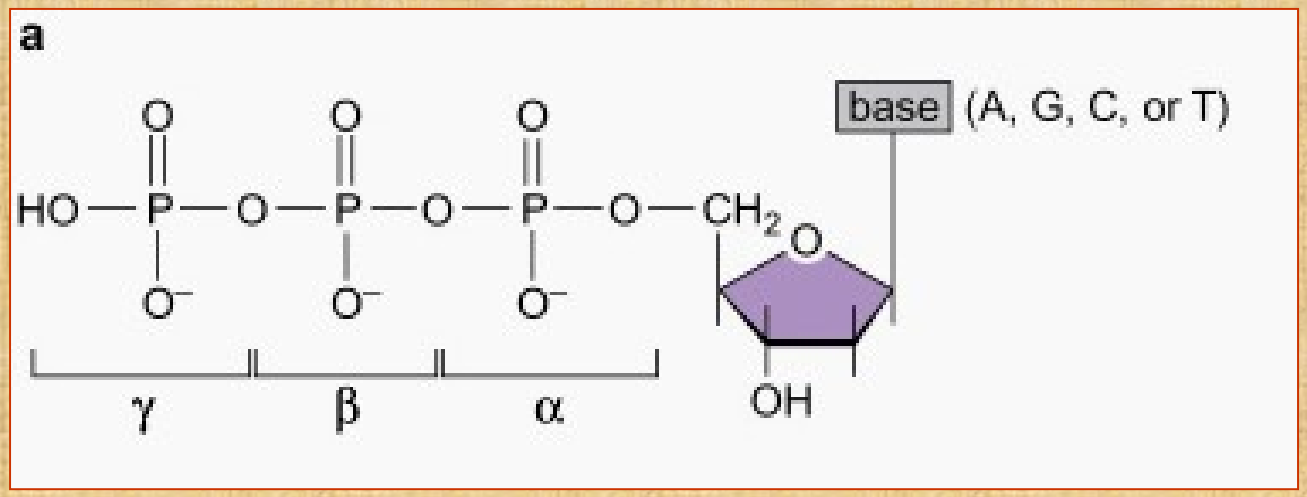


- **Cooperative binding**
- **Sequence-independent manner**

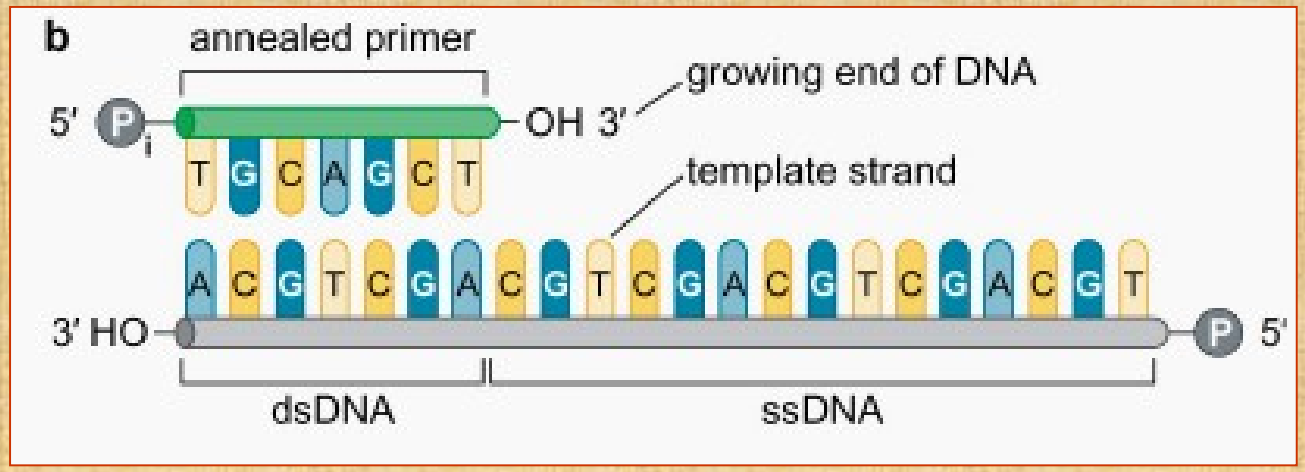
(electrostatic interactions)

SSB in E. coli and RPA in Eukaryotes

Substrates for DNA synthesis

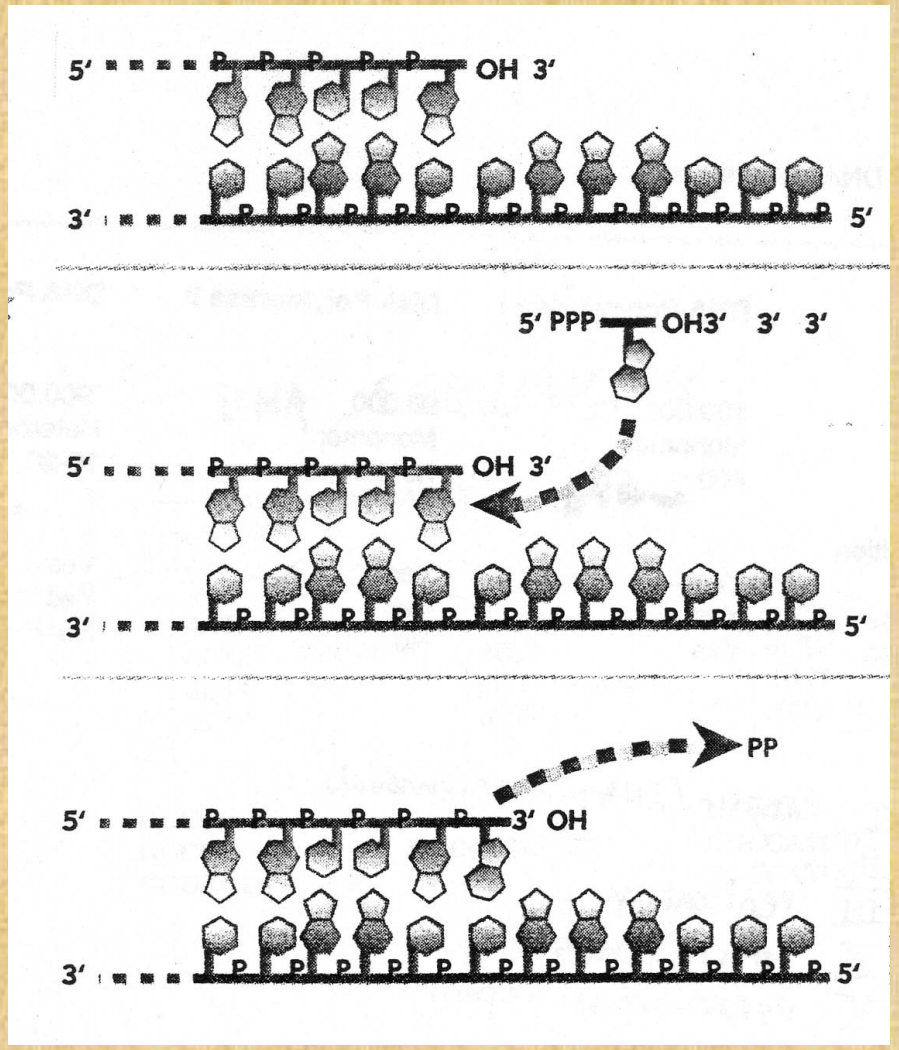


- dGTP, dCTP, dATP, & dTTP.
- dNTPs have three phosphoryl groups attached to 5' OH of 2' deoxyribose



- Primer template junction.
- Primer is a substrate & chemically modified.
- Template provides information

DNA is synthesized by Extending the 3’ End of t



Primer has a free 3’-OH

Incoming dNTP has a 5’ triphosphate

Equilibrium constant Keq = 10
DNA replication is irreversible

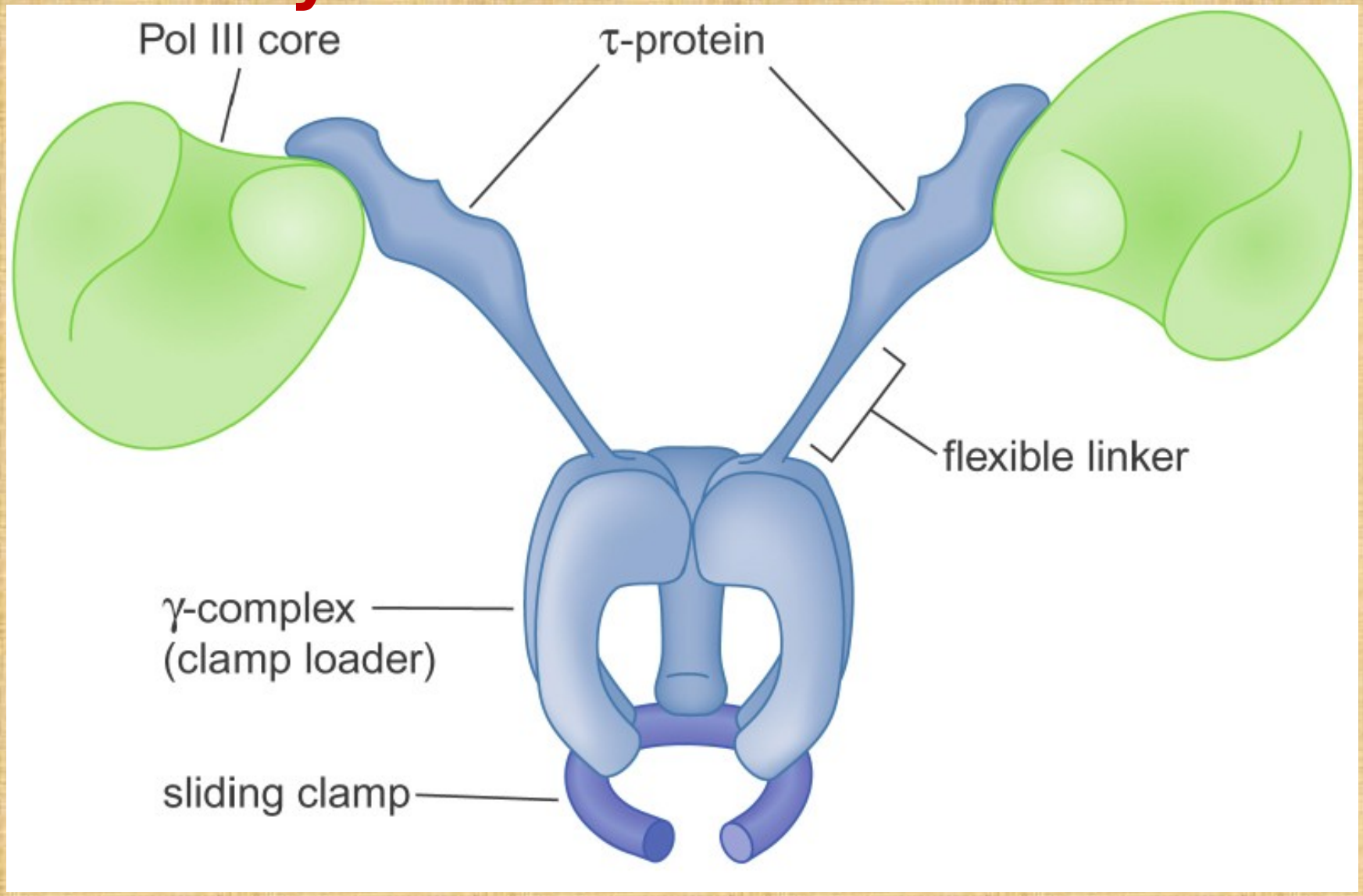
Pyrophosphate (PP) is lost when dNMP adds to the chain

$$XTP + (XMP)_n \rightarrow (XMP)_{n+1} + P{\sim}P \quad (\Delta G = -3.5 kcal/mole)$$

$$P{\sim}P \xrightarrow{\text{Pyrophosphatase}} 2Pi$$

$$XTP + (XMP)_n \rightarrow (XMP)_{n+1} + 2Pi \quad (\Delta G = -7.0 kcal/mole)$$

The composition of the DNA Pol III holoenzyme



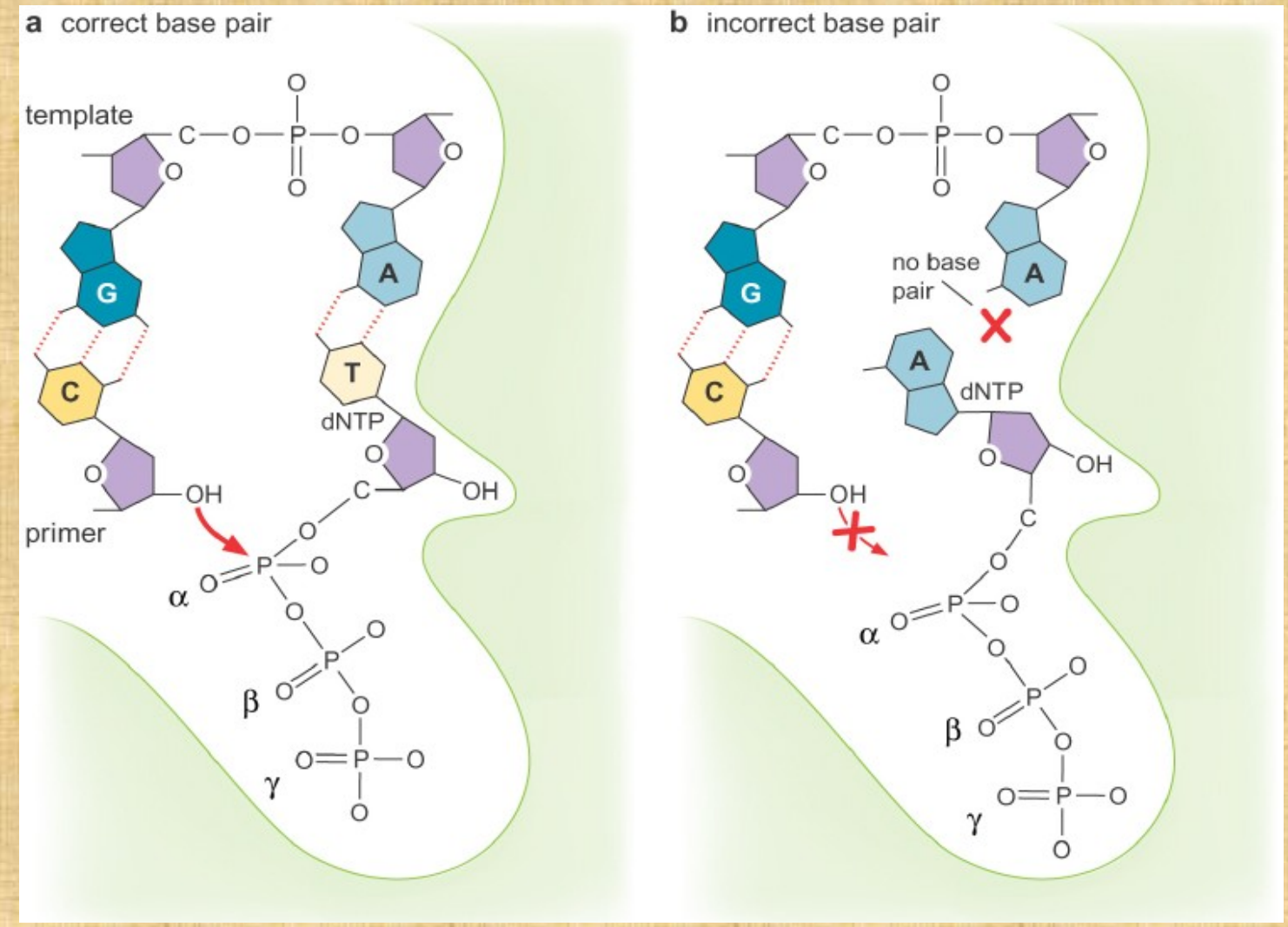
The mechanism of DNA Polymerase

DNA Pol uses a single active site to catalyze DNA synthesis

A single site to catalyze the addition of any of the four dNTPs.

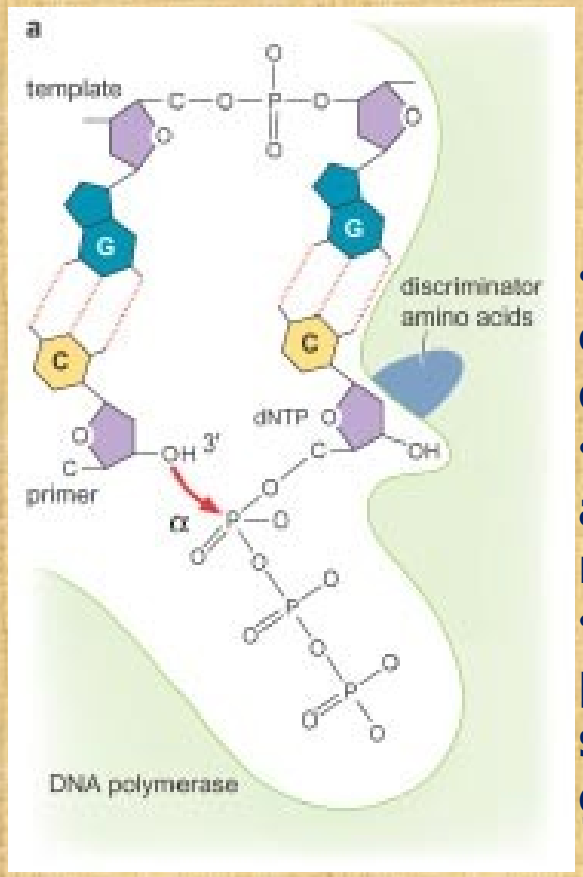
Recognition of different dNTP by monitoring the ability of incoming dNTP in forming A-T and G-C base pairs; incorrect base pair dramatically lowers the rate of catalysis (kinetic selectivity).

Distinguishing different dNTPs: kinetic selectivity

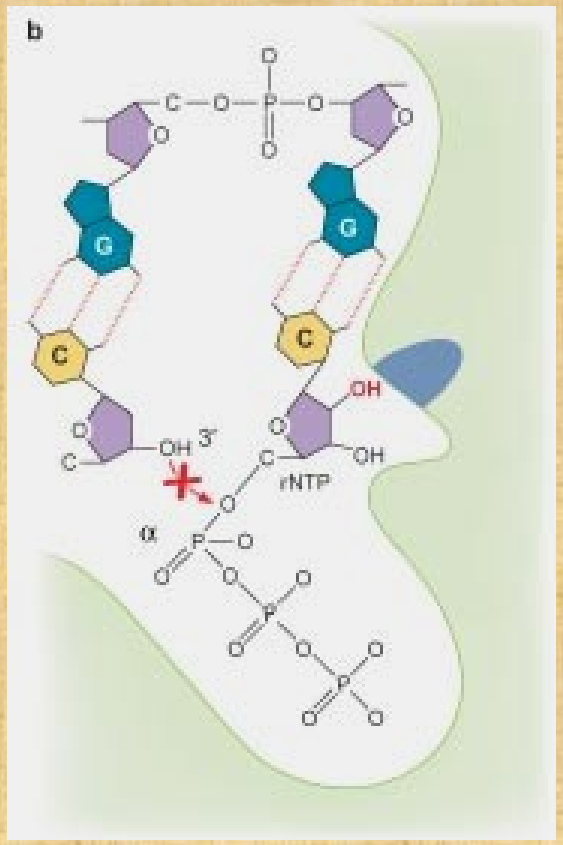


Rate of incorporation of wrong nucleotide is 10,000 fold much slower than incorporating a correct base.

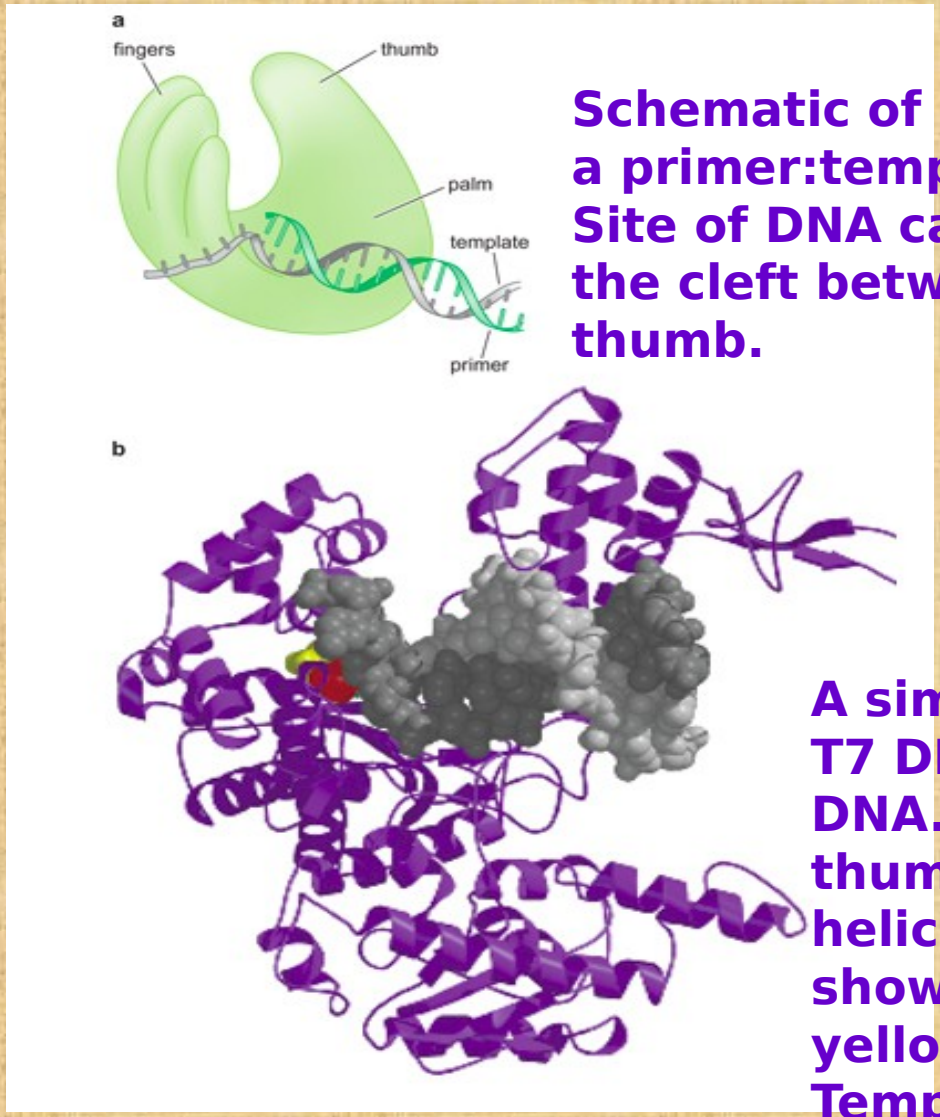
Distinguishing between rNTP and dNTP by steric exclusion of rNTPs from the active site.



- rNTPs -10 fold higher conc. in cell than dNTPs.
- rNTPs incorporated at a 1000 fold lower rate than dNTPs
- Nucleotide binding pocket in DNA pol too small to allow 2’ OH group of ribose.



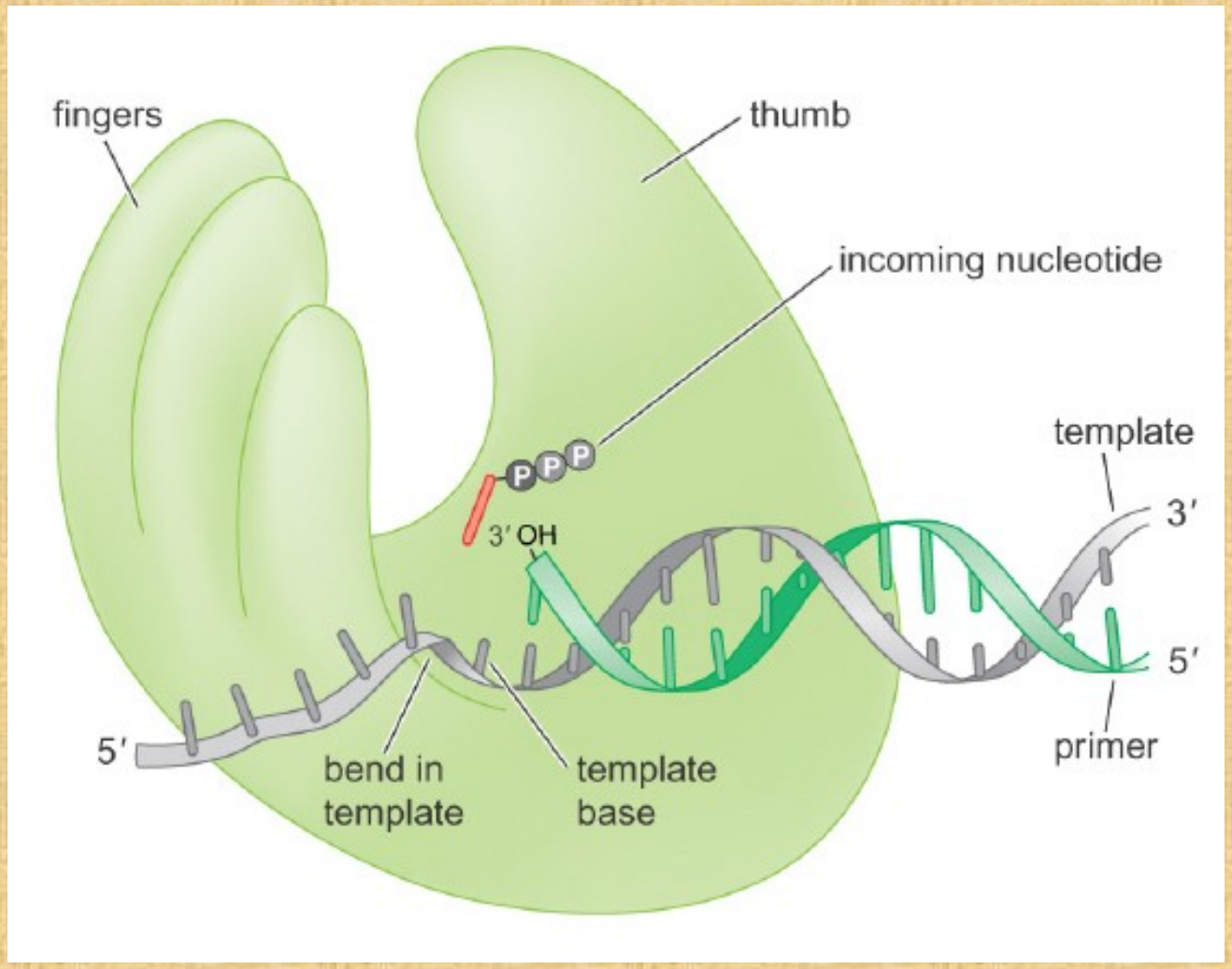
DNA Pol resemble a hand that grips the primer-template junction



Schematic of DNA pol bound to a primer:template junction. Site of DNA catalysis located in the cleft between fingers and thumb.

A similar view of the T7 DNA pol bound to DNA. Fingers and thumb composed of α helices. dNTP is shown in red and yellow (P-P-P). Template is dark grey and primer is light

Path of the Template DNA through the DNA Polymerase



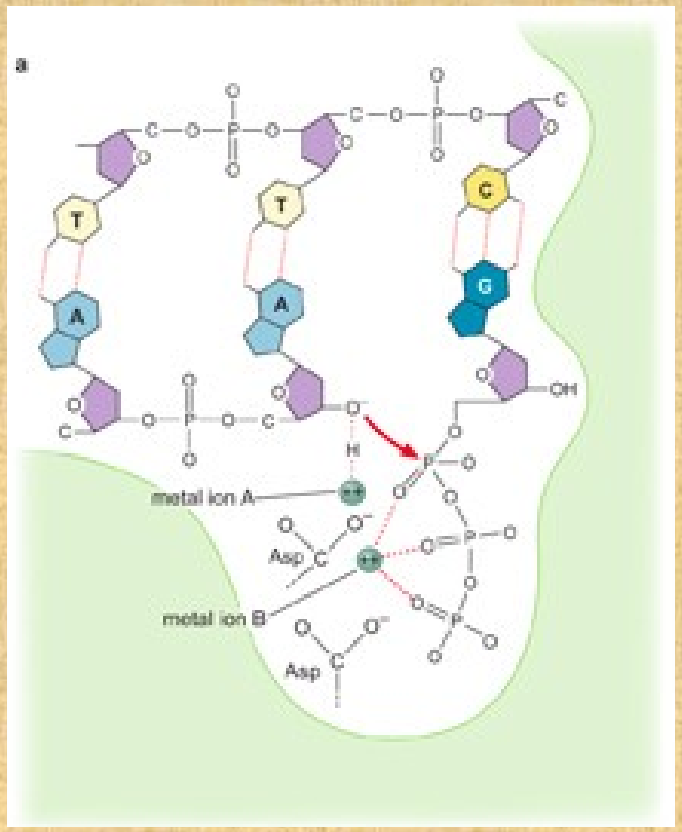
- Finger domain interacts with template strand leading to the 90° turn of PD backbone of template after active site.

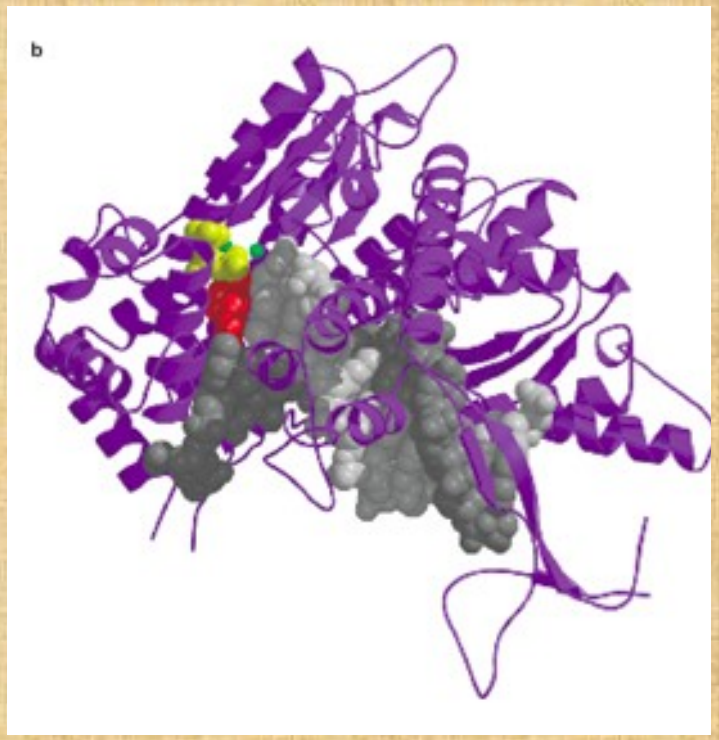
- This results in the second and all subsequent SS strand bases in a position that prevent any base pairing with a dNTP bound at the active site.

DNA Polymerase-palm domain

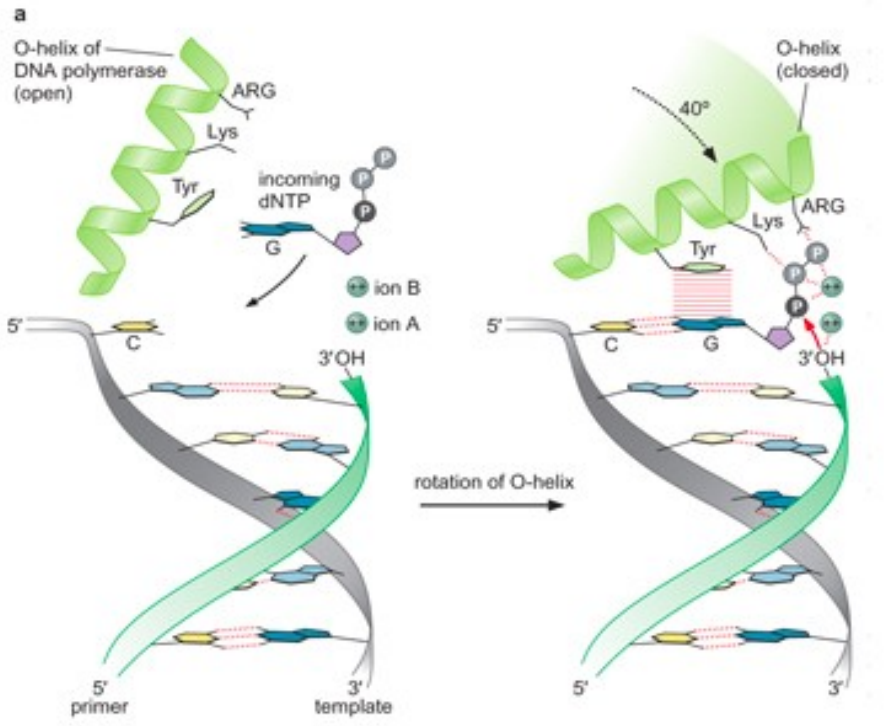
1. It is composed of a β sheet and contains two catalytic sites, one for addition of dNTPs and one for removal of the mis-paired dNTP.
2. The polymerization site: (1) binds to two metal ions Mg or Zn that alter the chemical environment around the catalytic site and lead to the catalysis. (2) Monitors the accuracy of base-pairing for the most recently added nucleotides by forming extensive hydrogen bond contacts with minor groove of the newly synthesized DNA.
3. Exonuclease site/proof reading site.

Two metal ions bound DNA polymerase catalyze nu





- **Two metal ions held in place by highly conserved two asp residues.**
- **Metal ion A interacts with 3'OH resulting in reduced association between O and H leaving a nucleophillic 3'O-.**
- **Metal ion B interacts with triphosphates of the incoming dNTP to neutralize their negative charge. Pyrophophate is**



DNA Polymerase-finger domain

Binds to the incoming dNTP, encloses the correct paired dNTP to the position for catalysis

Bends the template to expose the only nucleotide at the template that is ready for forming base pair with the incoming nucleotide

Stabilization of the pyrophosphate

DNA Polymerase-thumb domain

Not directly involved in catalysis instead interacts with the most recently synthesized DNA.

Interacts with the synthesized DNA to maintain correct position of the primer and the active site, and to maintain a strong association between DNA Pol and its substrate. Maintains high processivity.

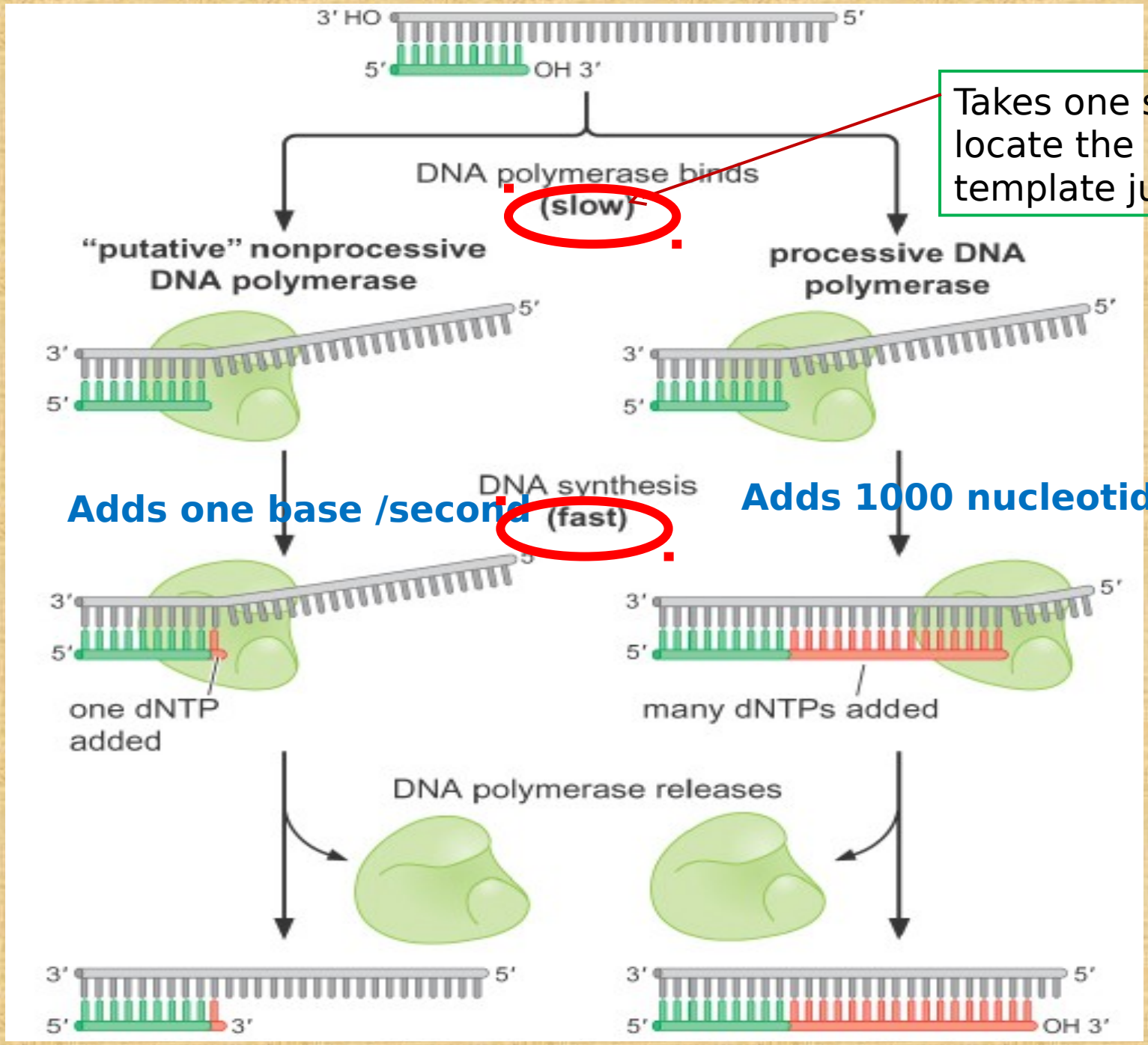
DNA Polymerases are Processive Enzymes

The rate of DNA synthesis is closely related to the polymerase processivity, because the rate-limiting step is the initial binding of polymerase to the primer-template junction.

Degree of processivity is defined as the average number of nucleotides added each time the enzyme binds a primer-template junction.

Processivity of DNA pol – from only a few nucleotides to > 50,000 added per binding event.

DNA polymerase binds to double stranded portion of the DNA in a sequence nonspecific manner. These interaction include electrostatic interactions between thumb domain and phosphate backbone and interactions of minor groove of DNA and palm domain.


3′ HO
5′
5′
OH 3′
DNA polymerase binds
(slow)
Takes one second to locate the primer-template junction
"putative" nonprocessive DNA polymerase
processive DNA polymerase
3′
5′
5′
3′
5′
5′
Adds one base /second
DNA synthesis
(fast)
Adds 1000 nucleotides/second
one dNTP added
many dNTPs added
DNA polymerase releases
3′
5′
5′
3′
3′
5′
5′
OH 3′

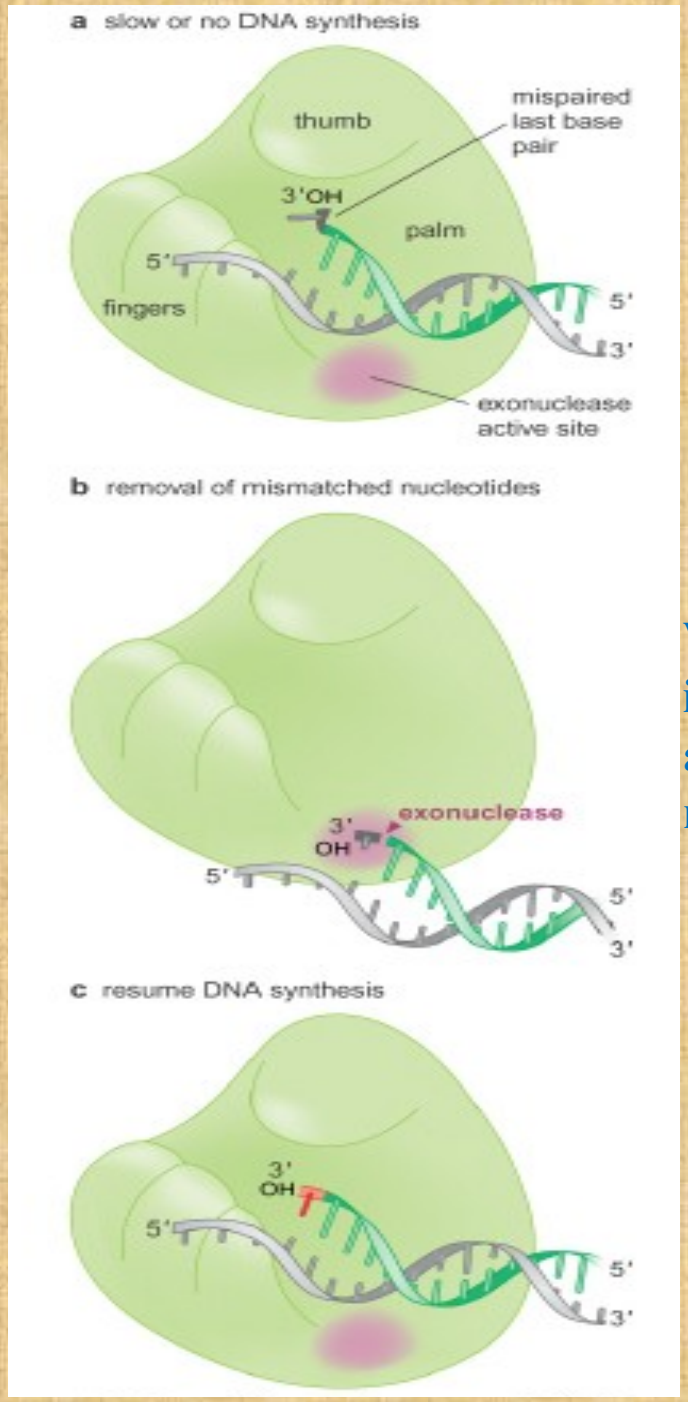
Exonucleases proofread newly synthesized DNA

The occasional flicking of the bases into "wrong" tautomeric form results in incorrect base pair and mis-incorporation of dNTP. (10^{-5} mistake)

The mismatched dNMP is removed by proofreading exonuclease, a part of the DNA polymerase.

Exonuclease activity allows one wrong base in every 10^{7} bases added.

A mismatched nucleotide decreases the rate of new nucleotide addition and incraese the rate of proofreading exonuclease activity.



When a wrong nucleotide is added into DNA, the rate of DNA synthesis is reduced and the affinity of the 3' end of the primer for DNA polymerase active site is diminished.

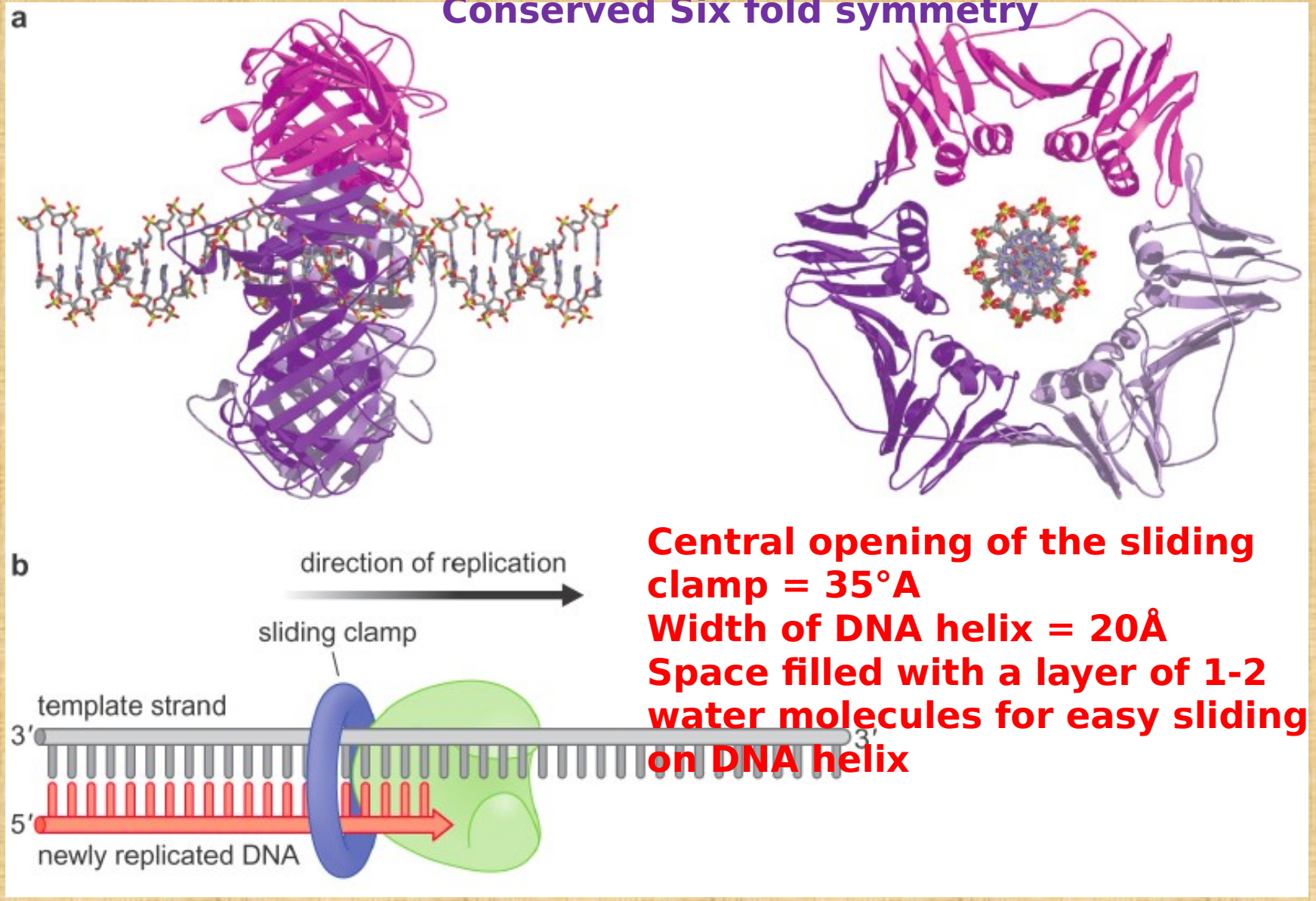
When mismatched, the 3' end of the DNA has increased affinity for the proofreading exonuclease active site. Once bound at the active, the mismatched nucleotide is removed.

Once the mismatched nucleotide is removed, the affinity of the properly base paired DNA for the DNA polymerase active site is restored and DNA synthesis continues

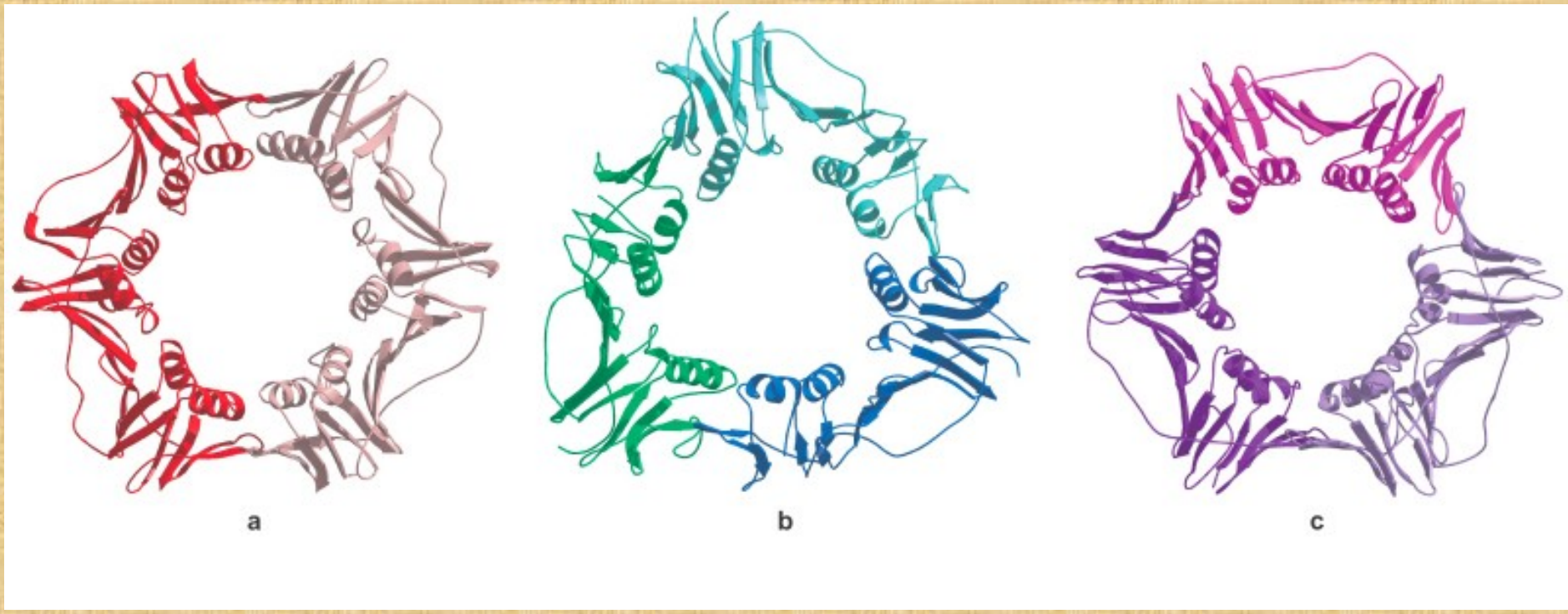
Sliding clamps dramatically increase DNA polymerase activity and processivity

- **Encircle** the newly synthesized double-stranded DNA and the polymerase associated with the primer:template junction
- In the absence of sliding clamp, DNA pol diffuses away every 50-100 bases synthesized.
- **Ensures** the rapid rebinding of DNA Pol to the same primer:template junction, and thus increases the processivity of Pol.
- Eukaryotic sliding DNA clamp is PCNA (Proliferating Cell Nuclear Antigen)

Conserved Six fold symmetry



Central opening of the sliding clamp = 35°A
Width of DNA helix = 20Å
Space filled with a layer of 1-2 water molecules for easy sliding on DNA helix



a) 3D structure of sliding clamp from *E.coli* is composed of two

b) 3 D structure of T4 phage sliding clamp is a trimer of the gp4

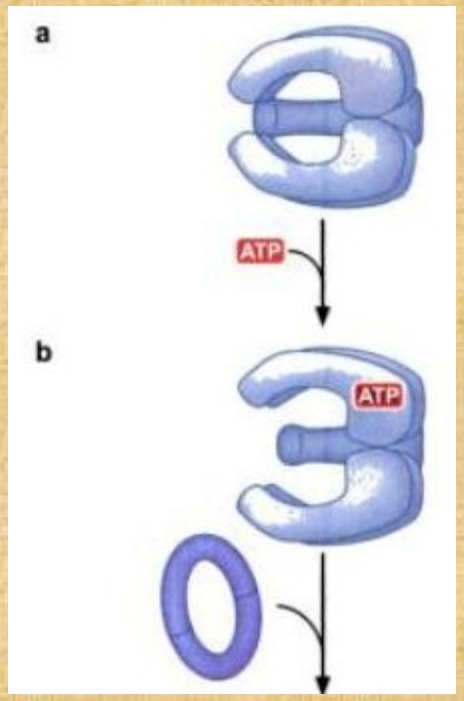
c) 3D structure of eukaryotic sliding clamp a trimer of the PCNA

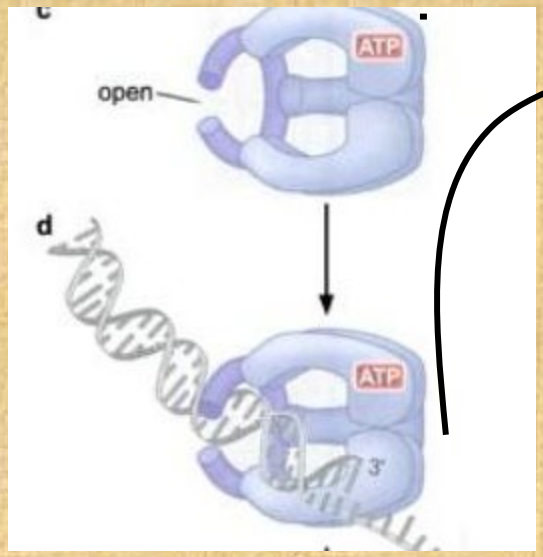
Sliding clamps are opened and placed on DNA by clamp loaders

- Clamp loader is a special class of protein complex catalyzes the opening and placement of sliding clamps on the DNA, such a process occurs anytime a primer:template junction is present.
- It is a single copy five protein γ-complex binds to both copies of the core DNA pol III.
- Sliding clamps are only removed from the DNA once all the associated enzymes complete their function.
- DNA pol III holoenzyme – a general name for a multiprotein complex in which a core enzyme activity is associated with additional components that enhance function

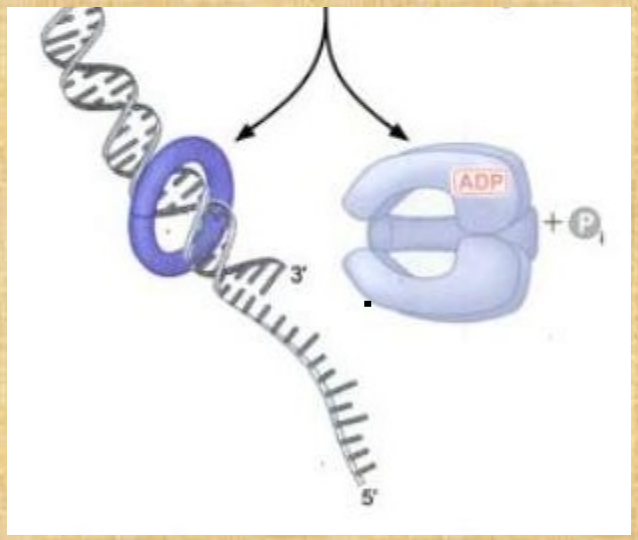
ATP control of sliding clamp loa

- Clamp loader can only bind the sliding clamp and DNA when bound to ATP
- Hydrolysis causes the clamp loader to release the sliding clamp and disassociate from the DNA.
- Once released from the clamp loader, the sliding clamp closes around the DNA
- ATP control of these events is most directly related to controlling the timing of conformational changes by the enzyme.





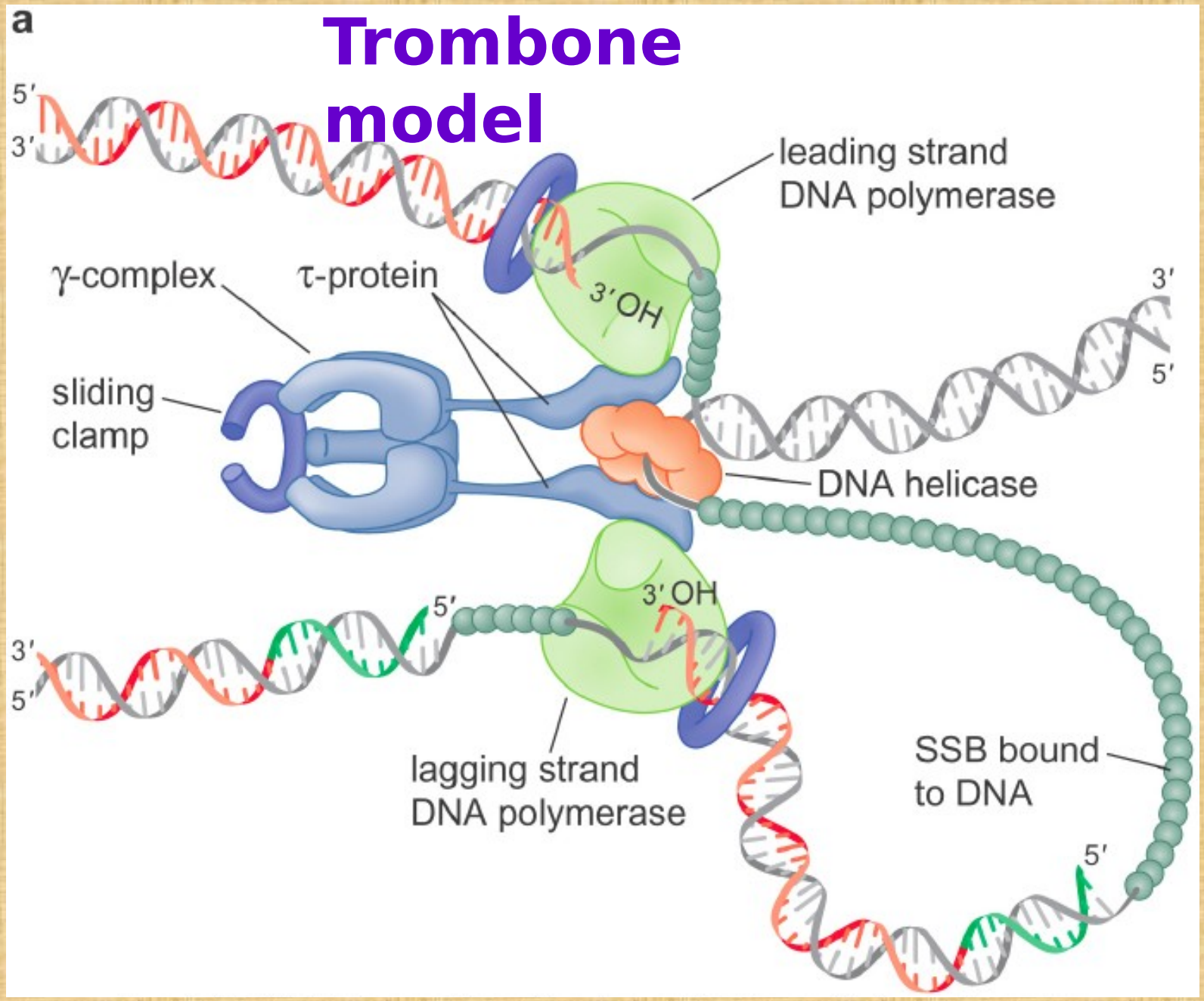
ATP hydrolysis

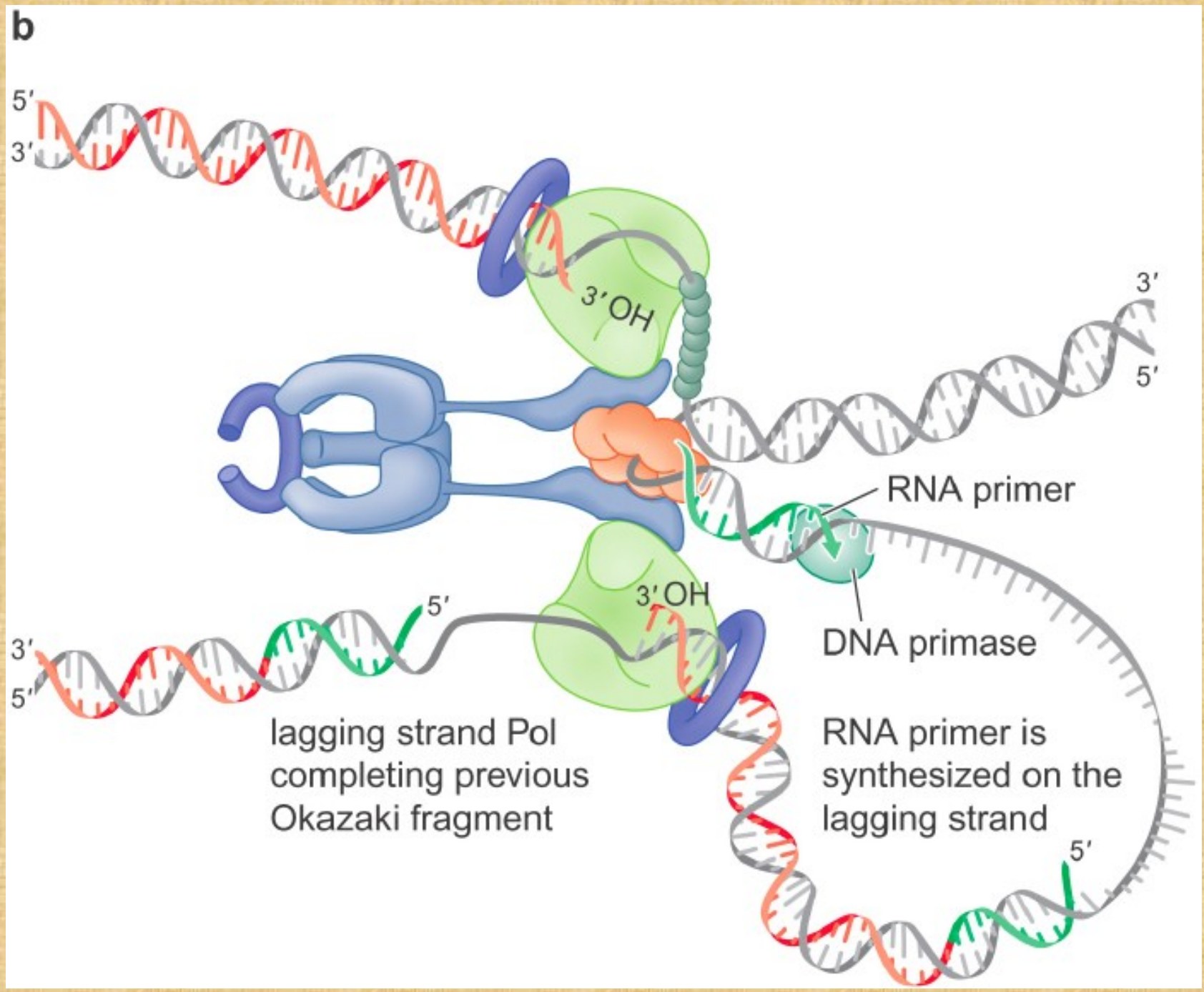


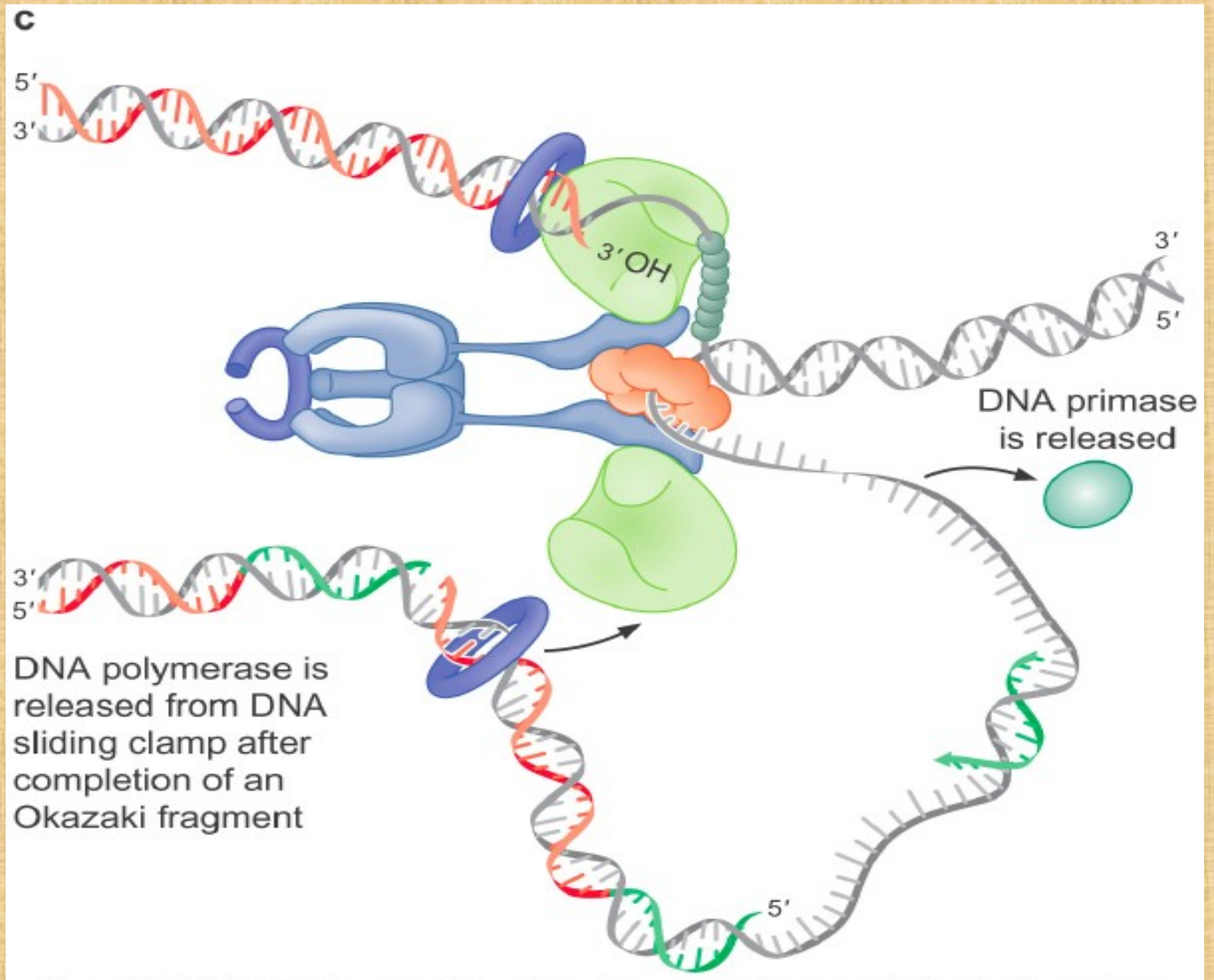
DNA Pols are specialized for different roles in the cell

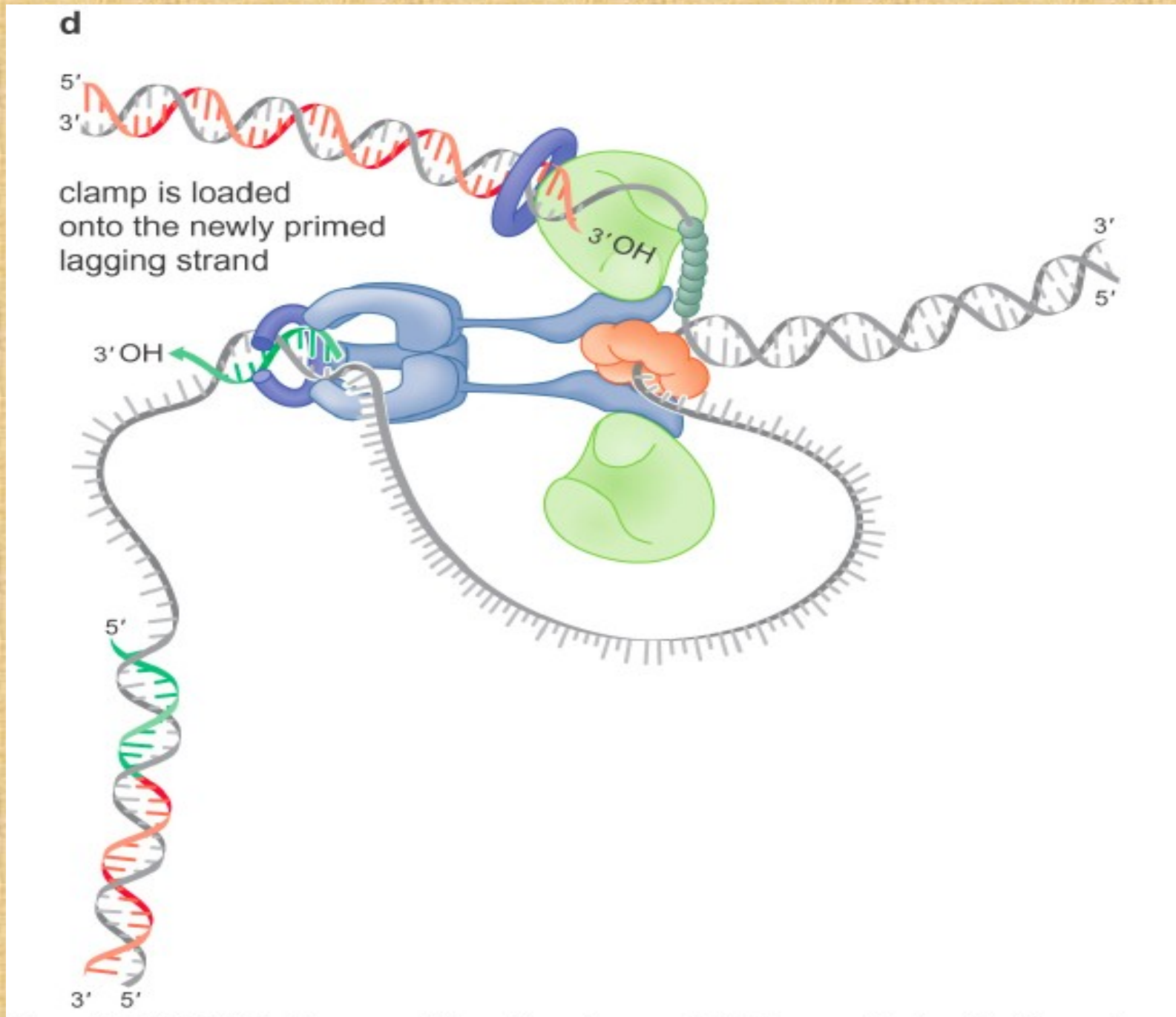
- Each organism has a distinct set of different DNA Pols
- Different organisms have different DNA Pols.
- DNA Pol III holoenzyme: a protein complex responsible for *E. coli* genome replication.
- DNA Pol I: removes RNA primers in *E. coli*
- Eukaryotic cells have multiple DNA polymerases. Three are essential to duplicate the genome: DNA Pol δ, DNA Pol εand DNA Pol α/primase.
- Polymerase switching in Eukaryotes: the process of replacing DNA Pol α/primase with DNA Pol δ or DNA Pol ε.

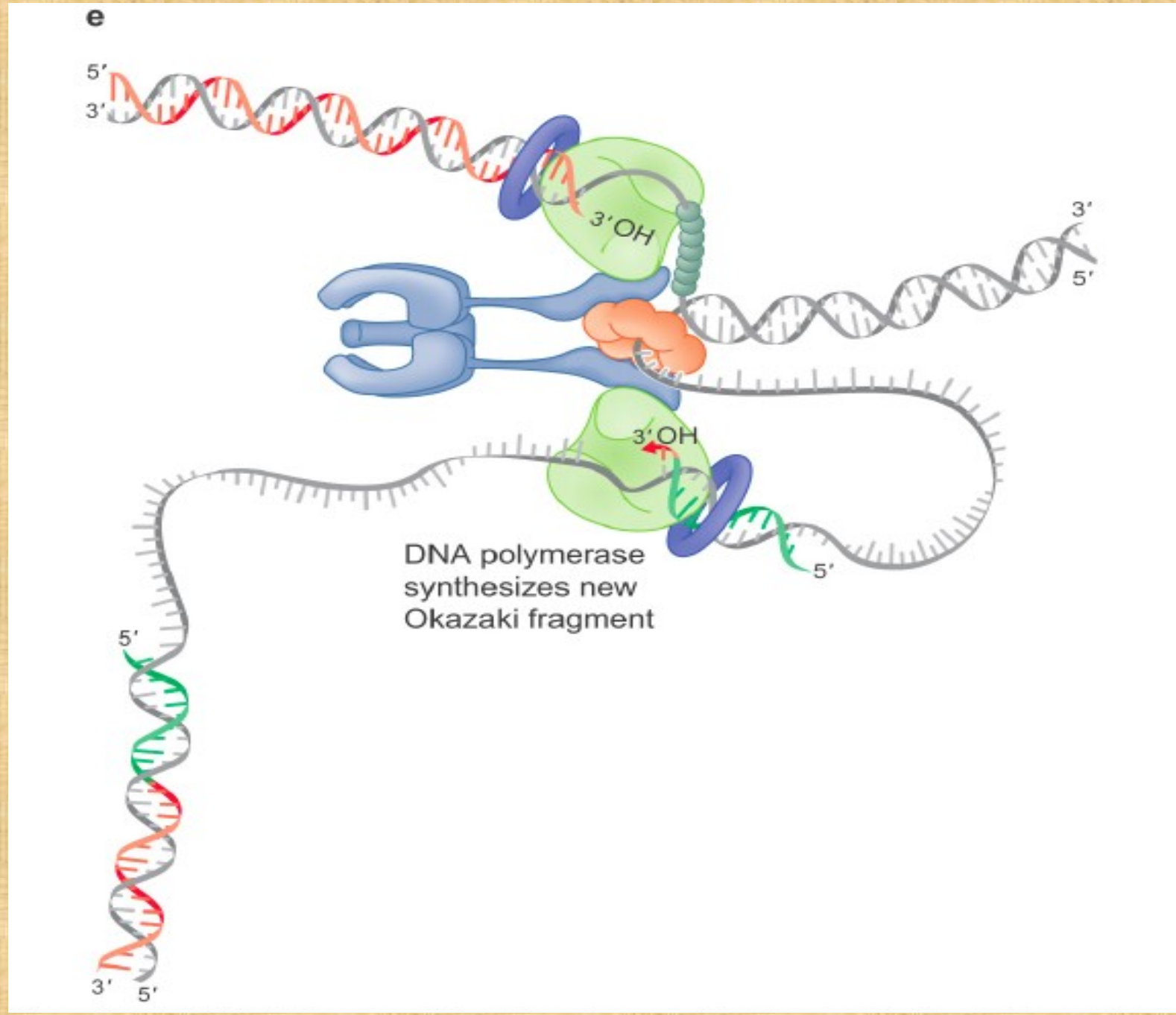
Trombone model




b
5′
3′
3′OH
3′
5′
RNA primer
3′OH
5′
3′
5′
DNA primase
lagging strand Pol
completing previous
Okazaki fragment
RNA primer is
synthesized on the
lagging strand
5′


c
5′
3′
3′OH
3′
5′
DNA primase
is released
3′
5′
DNA polymerase is
released from DNA
sliding clamp after
completion of an
Okazaki fragment
5′


d
5′
3′
clamp is loaded
onto the newly primed
lagging strand
3′OH
3′
5′
3′OH
5′
3′
5′


e
5′
3′
3′OH
3′
5′
3′OH
DNA polymerase
synthesizes new
Okazaki fragment
5′
5′
3′
5′

Interactions between replication fork proteins form the *E. coli* replisome

The combination of all proteins that function at the replication fork is referred to as replisome

Replisome is established by protein-protein interactions

1. **DNA helicase & DNA Pol III holoenzyme, this interaction is mediated by the clamp loader and stimulates the activity of the helicase (10-fold)**
2. **DNA helicase & primase, which is relatively week and strongly stimulates the primase function (1000-fold). This interaction is important for regulation the length of Okazaki fragments.**
3. **DNA Pol III holoenzyme, helicase and primase interact with each other to form replisome, a finely tuned factory for DNA synthesis with the activity of each protein is highly coordinated.**

Comparison of DNA Polymerases of *E. coli*

	DNA polymerase		
	I	II	III
Structural gene*	*pol*A	*pol*B	*pol*C (*dna*E)
Subunits (number of different types)	1	≥4	≥10
M_r	103,000	88,000†	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: mutagenesis (din B, subunit-1)
+ DNA pol V: error-prone repair (UmuC,UmuD’2, subunit-3)

Examples of eukaryotic DNA polymerases

Greek name	HUGO name	Class	Other names	Proposed main function
α (alpha)	POLA	B	*POL1*	DNA replication
β (beta)	POLB	X		Base excision repair
γ (gamma)	POLG	A	*MIP1*	Mitochondrial replication
δ (delta)	POLD1	B	*POL3*	DNA replication
ε (epsilon)	POLE	B	*POL2*	DNA replication
ζ (zeta)	POLZ	B	*REV3*	Bypass synthesis
η (eta)	POLH	Y	*RAD30*, XPV	Bypass synthesis
θ (theta)	POLQ	A	mus308, eta	DNA repair
ι (iota)	POLI	Y	RAD30B	Bypass synthesis
κ (kappa)	POLK	Y	DinB1, theta	Bypass synthesis
λ (lambda)	POLL	X	*POL4*, beta2	Base excision repair
μ (mu)	POLM	X		Non-homologous end joining
σ (sigma)	POLS	X	*TRF4*, kappa	Sister chromatid cohesion
	REV1L	Y	*REV1*	Bypass synthesis
	TDT	X		Antigen receptor diversity

plus many more

Pol α β δ ε γ (mitochondrial)

Mass 300,000 40,000 170-230,000 250,000 180-300,000

Ligase

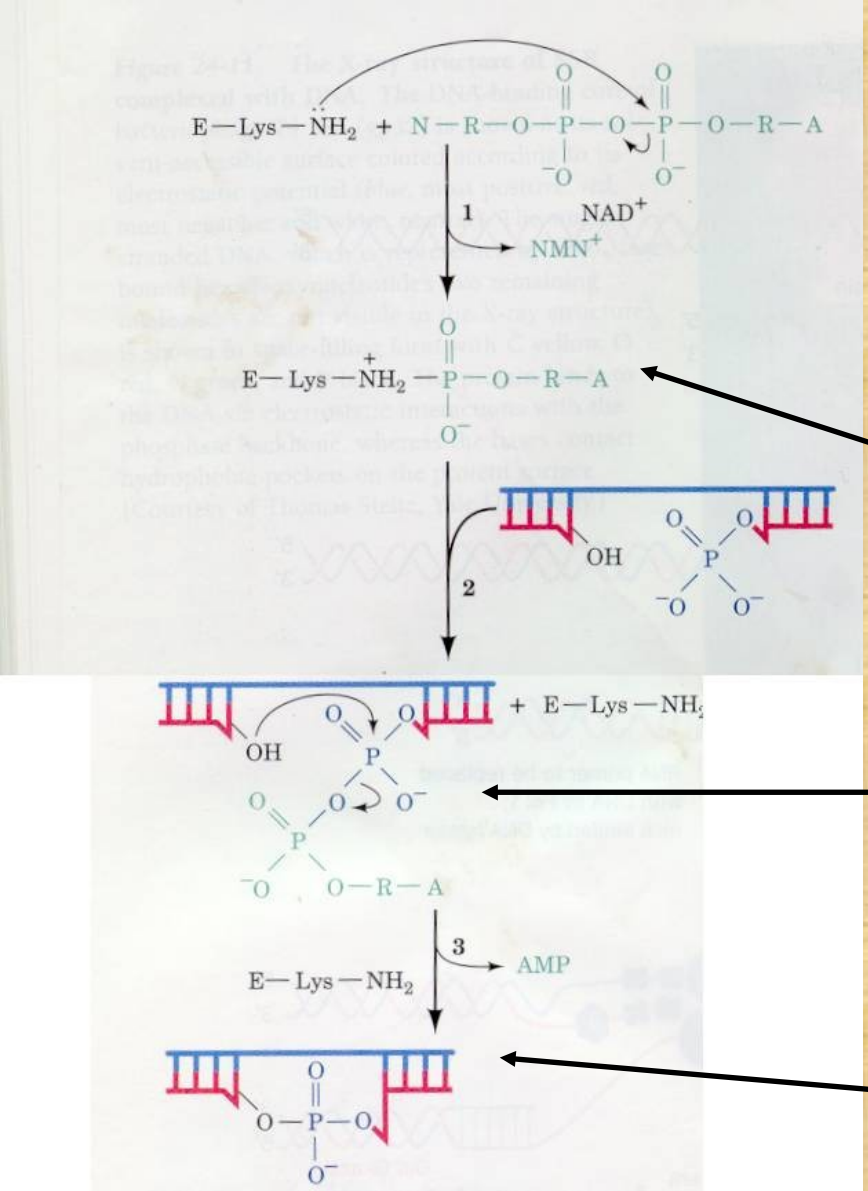
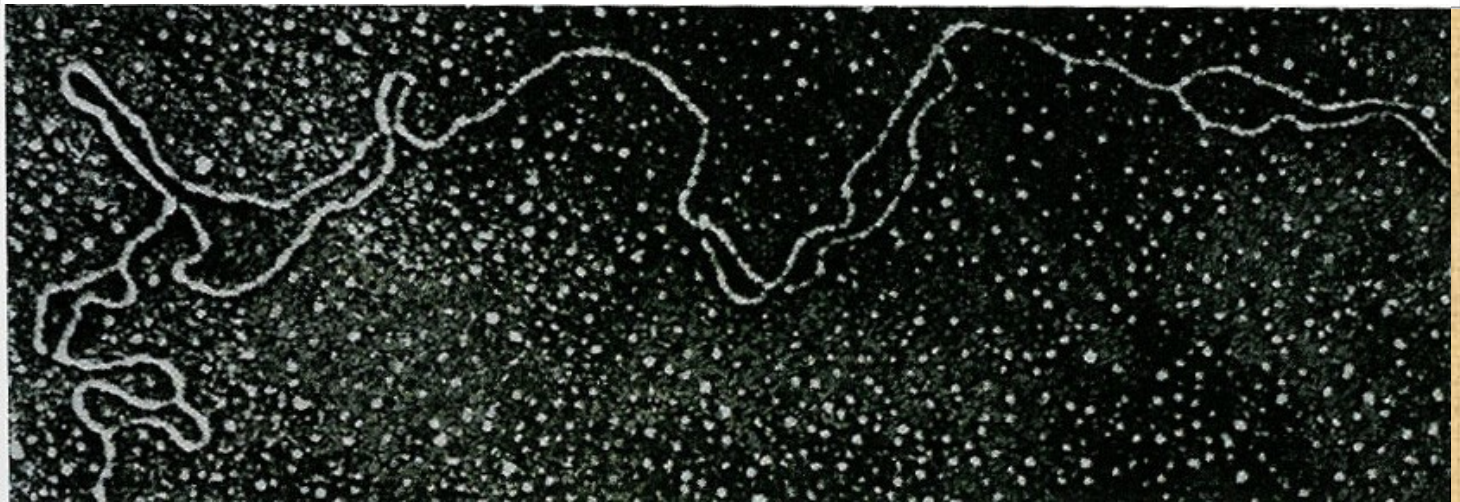


Figure 24-13. **The reactions catalyzed by *E. coli* DNA ligase.** In eukaryotic and T4 ligases, NAD^+ is replaced by ATP so that PP_i rather than NMN^+ is eliminated in the first reaction step. Here A, R, and N represent the adenine, ribose, and nicotinamide residues, respectively.

- Uses NAD^+ or ATP for coupled reaction
- 3-step reaction:
  - AMP is transferred to Lysine residue on enzyme
  - AMP transferred to open 5’ phosphate via temporary pyrophosphate (i.e., activation of the phosphate in the nick)
  - AMP released, phosphodiester linkage made
- NAD□NMN + AMP

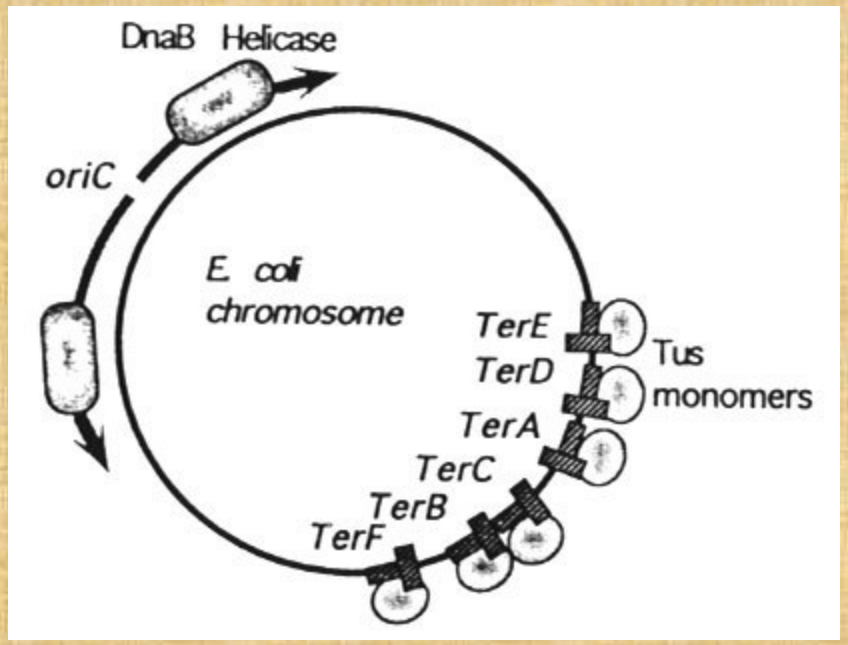
Eukaryotes need multiple replication origins

Genome	Fork speed	S phase	Origins	Comment
		S phase = DNA Synthesis		
E. coli	4.6 Mbp	30 kb/min	40 min	1	
Yeast	14 Mbp	3 kb/min	20 min	~330	S would last 80 hr if 1 ori
	1 L culture = 4.10^{10} cells --> 400 000 km DNA synthesized (Earth-Moon distance)			
Human	3 Gbp	3 kb/min	7 h	>10 000 ?	S would last 1 year if 1 ori
	2.10^{13} km DNA synthesized (2 light-years) during life time (10^{16} cell divisions)			



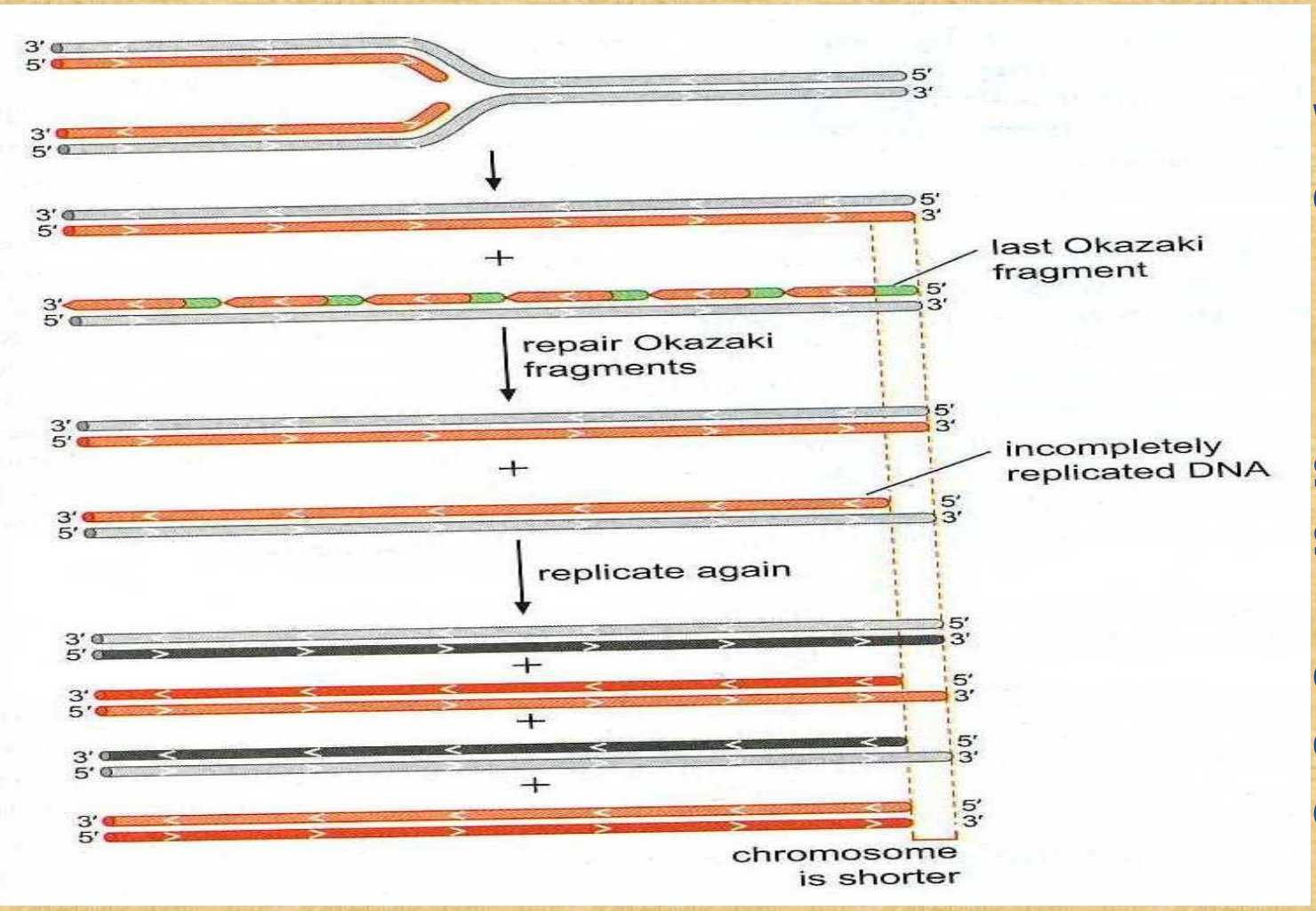
Replication Termination in Prokaryotes

- Termination sites are labeled TerA through TerF.
- The sites are located between 23 and 29 min (TerA, TerD, and TerE), 33 and 36 min (TerB and TerC), and at 48 min (TerF); thus these sites are spread over a long distance (1 min is approximately equal to 50 kb).
- The T shape of the termination site denotes the polarity of the site; replication forks meeting the flat side (top of the T) are arrested (that is, the clockwise fork will pass through sites TerE, TerD, and TerA, but will stop at TerC, TerB, or TerF).
- A protein called Tus binds to the Ter sites, and this binding stops DnaB (helicase) action.



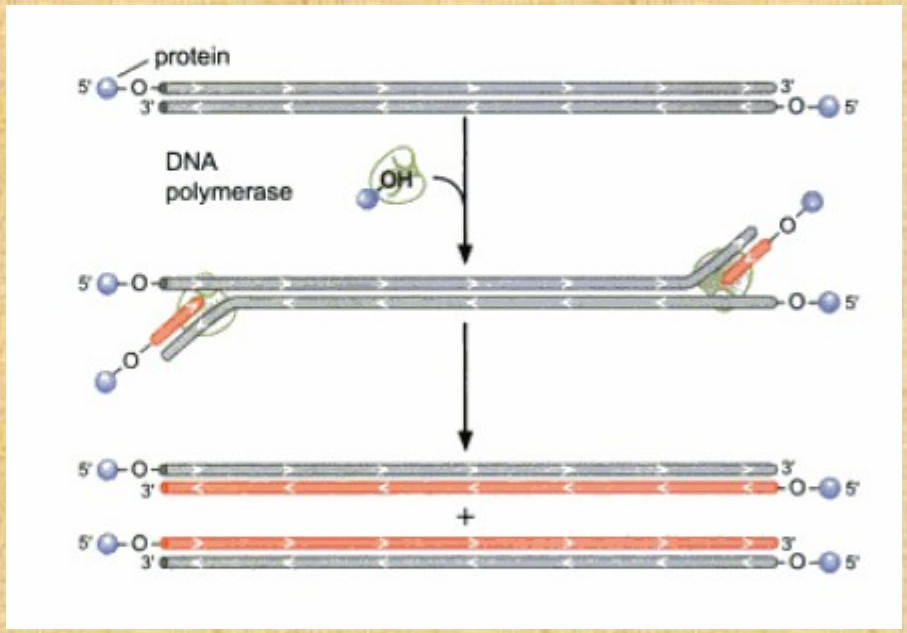
Finishing replication in eukaryotes:

The end replication problem
Telomere & telomerase: a link with cancer and agi



What is the end replication problem? Lagging strand synthesis is unable to copy the extreme ends of the linear

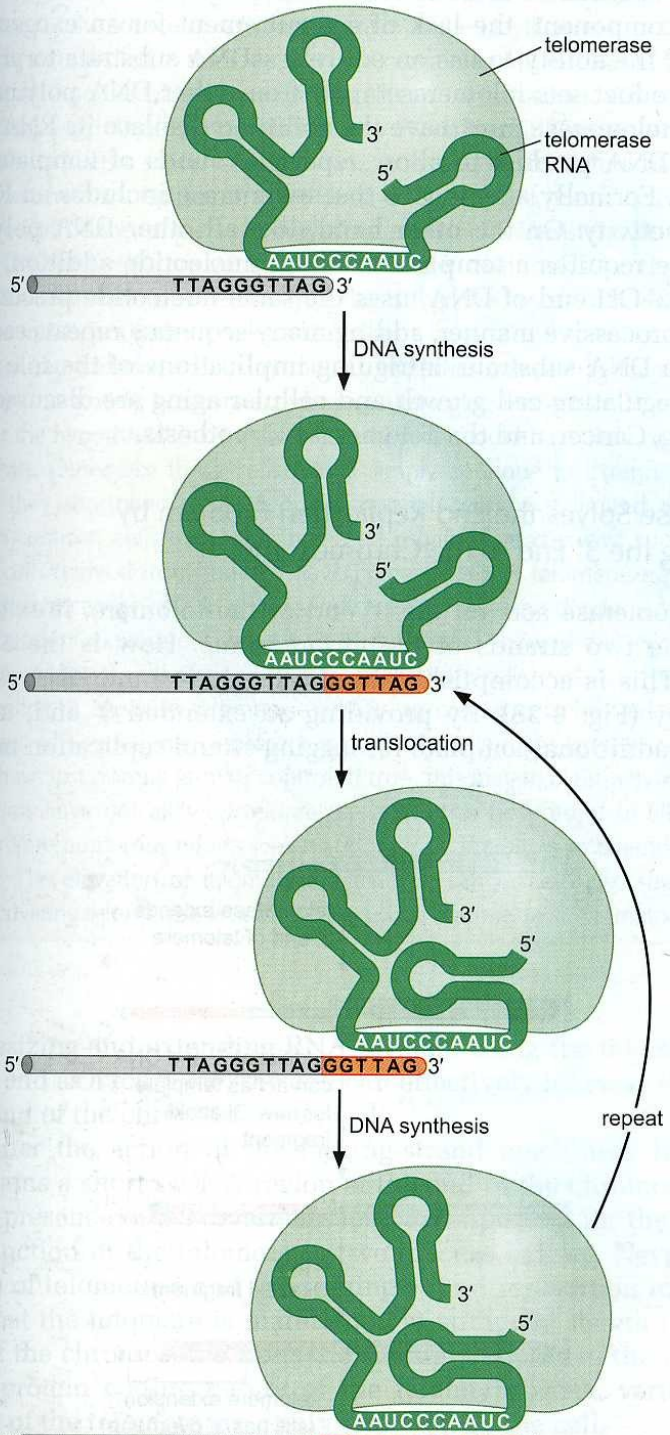
Protein priming as a solution to the end replication problem



The priming protein binds to the lagging strand template and uses an amino acid to provide OH .

Priming protein becomes covalently linked to the 5'end of the chromosome

Happens in bacteria with linear chromosomes and viruses



How telomerase works?

Telomeres – head to tail repeats of a sequence 5'TTAGGG-3'.

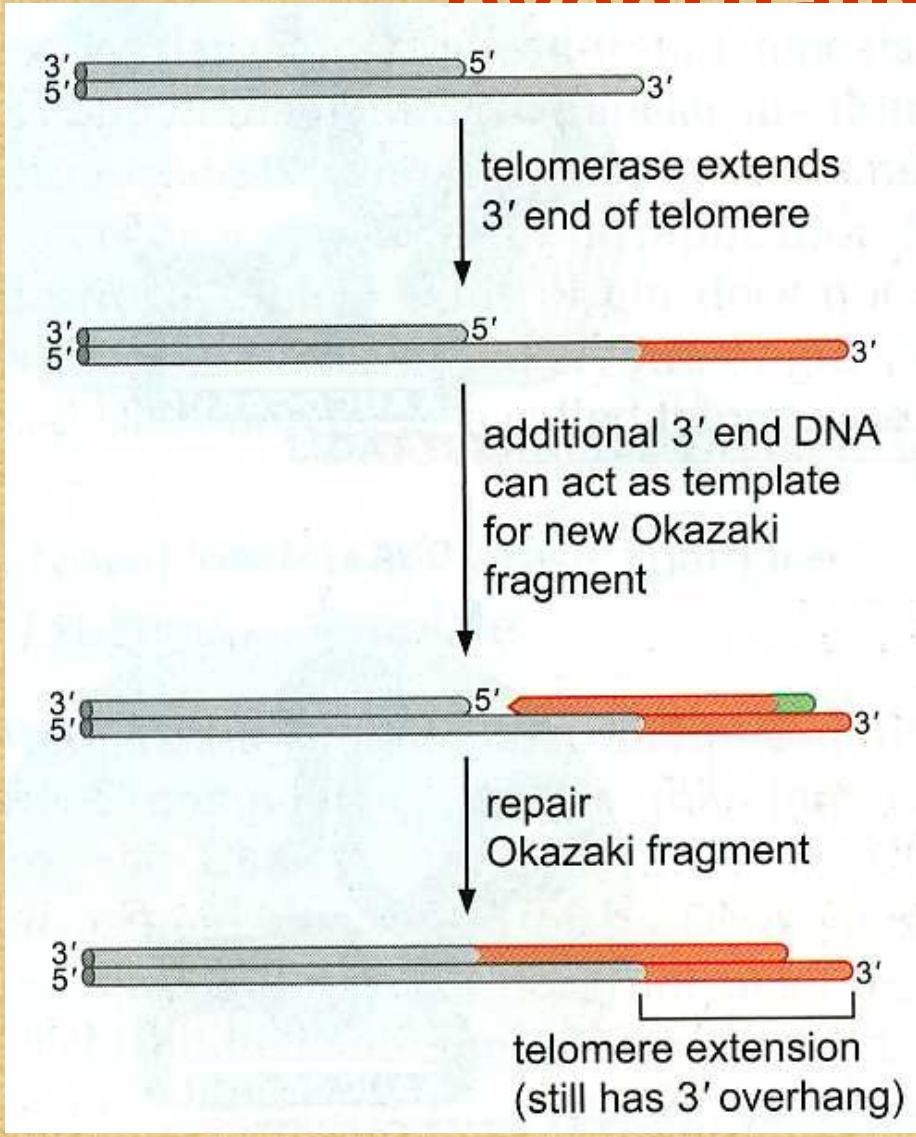
Telomerase consists of a protein reverse transcriptase and an RNA

It extends the protruding 3' end of the chromosome using its RNA components as a template.

Telomerase was discovered by Carol W. Greider and Elizabeth Blackburn in 1984 in the ciliate *Tetrahymena*. Together with Jack W. Szostak, Greider and Blackburn were awarded the 2009 Nobel Prize in Physiology or Medicine for their discovery.

The sequence of RNA in telomerase has 1.5 copies 3'-CAAUCCCAAUC-5' **complementary to teleomere.**

How the end problem is eventually resolved?



The extended 3' end allows the DNA polymerase to synthesize a new Okazaki fragment, which prevents the loss of genetic information at the chromosomal end.