Replication

Replication

- Replication is a faithful duplication process of DNA or a polymerization reaction, and can be divided into initiation, elongation and termination.
- Genetic information is transferred from parent to progeny by replication.
- DNA is a double-helical molecule
- Watson and Crick Predicted Semi-conservative Replication of DNA

Replication

- **Overview**
- The mechanism for all replicating systems is **specificity of base pairing** – adenine with thymine and cytosine with guanine.
- Nucleotide monomers are added one by one to the end of a growing strand by an enzyme called DNA polymerase.
- The sequence of bases in each new or daughter strand is complementary to old or parent strand.

Basic Rules Of Replication

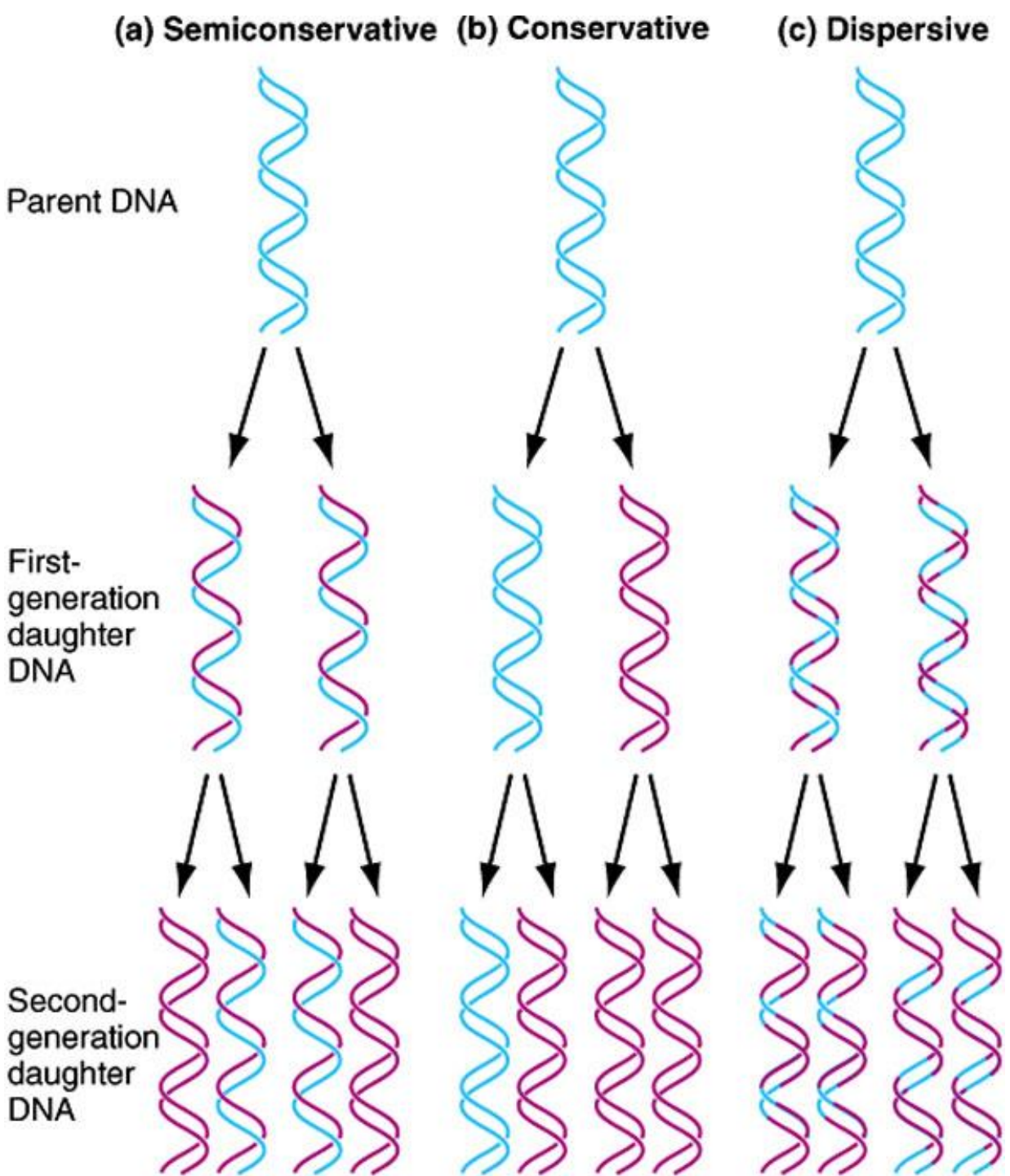
A. Semi-conservative
B. Starts at the ‘origin’
C. Synthesis always in the 5-3’ direction
D. Can be uni or bidirectional
E. Semi-discontinuous
F. RNA primers required

Semi-conservative Replication of DNA

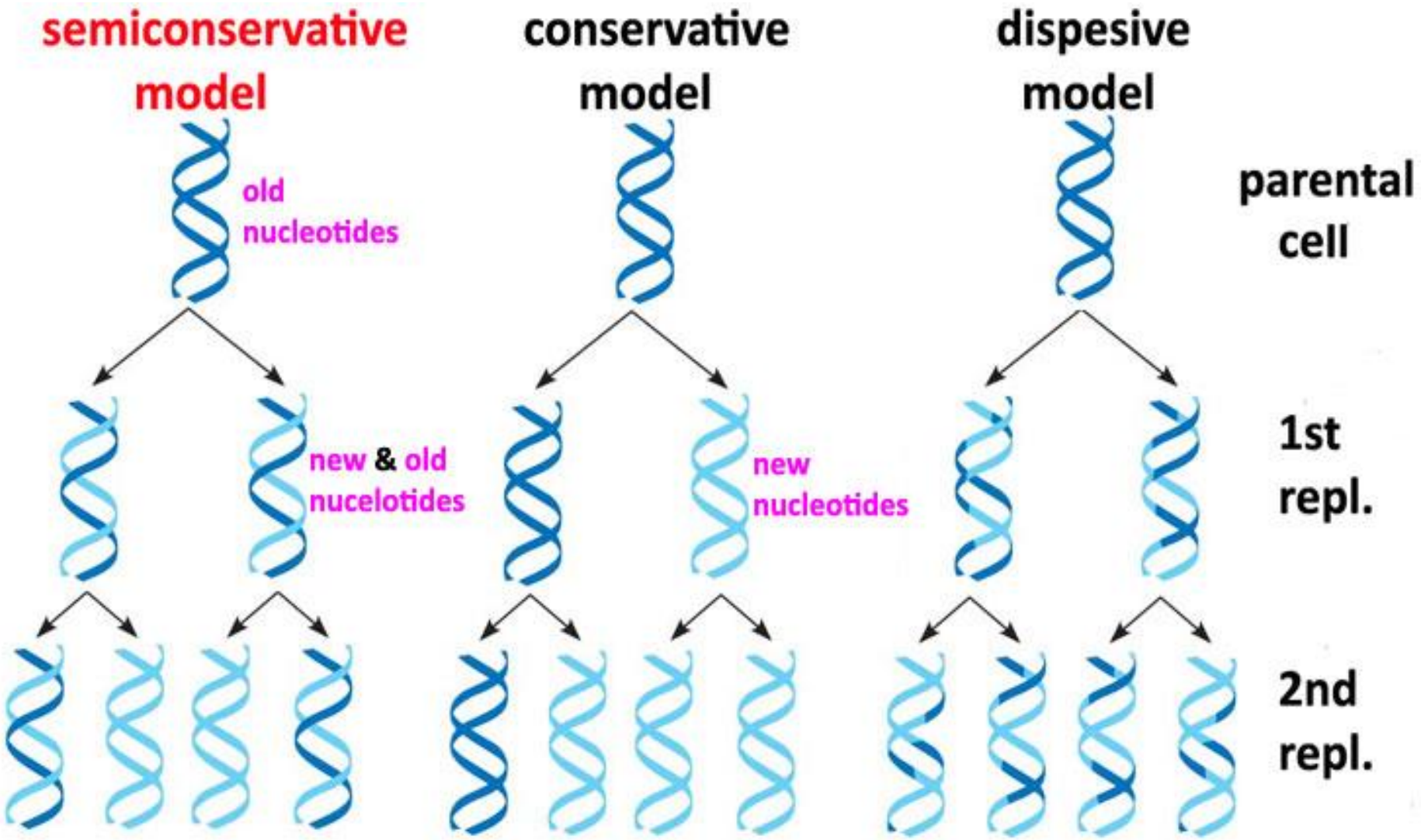
- The mechanism: Strand separation, followed by copying of each strand.
- Each separated strand acts as a template for the synthesis of a new complementary strand.
- Each strand of the helix must be copied in complementary fashion by DNA polymerase
- DNA replication takes place in a semiconservative manner.
- That is, a parent double helix forms two daughter double helices, each composed of one parent DNA stand and on newly synthesized stand.

DNA replication

3 possible models

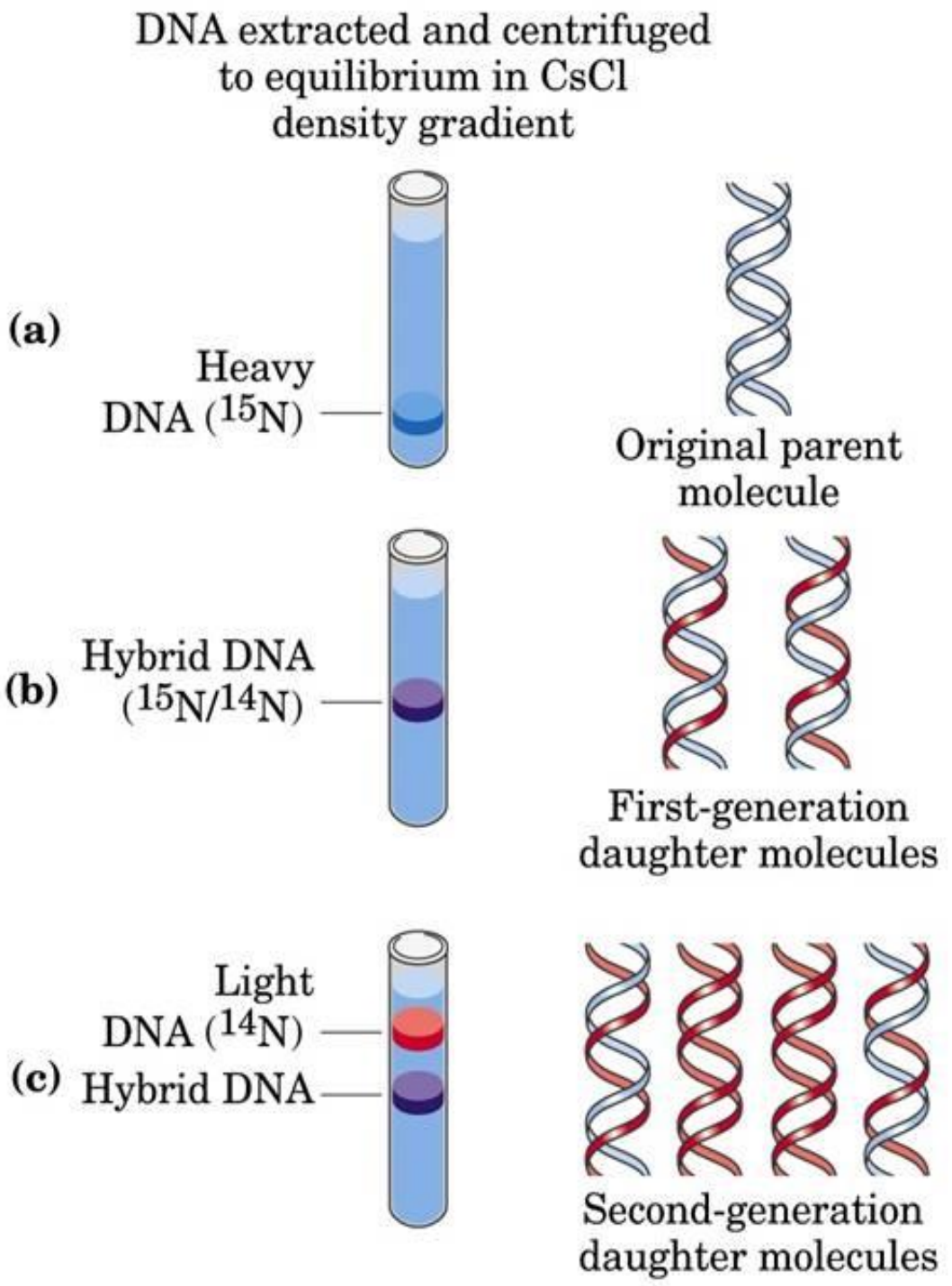


DNA replication - 3 possible models



Semiconservative DNA Replication

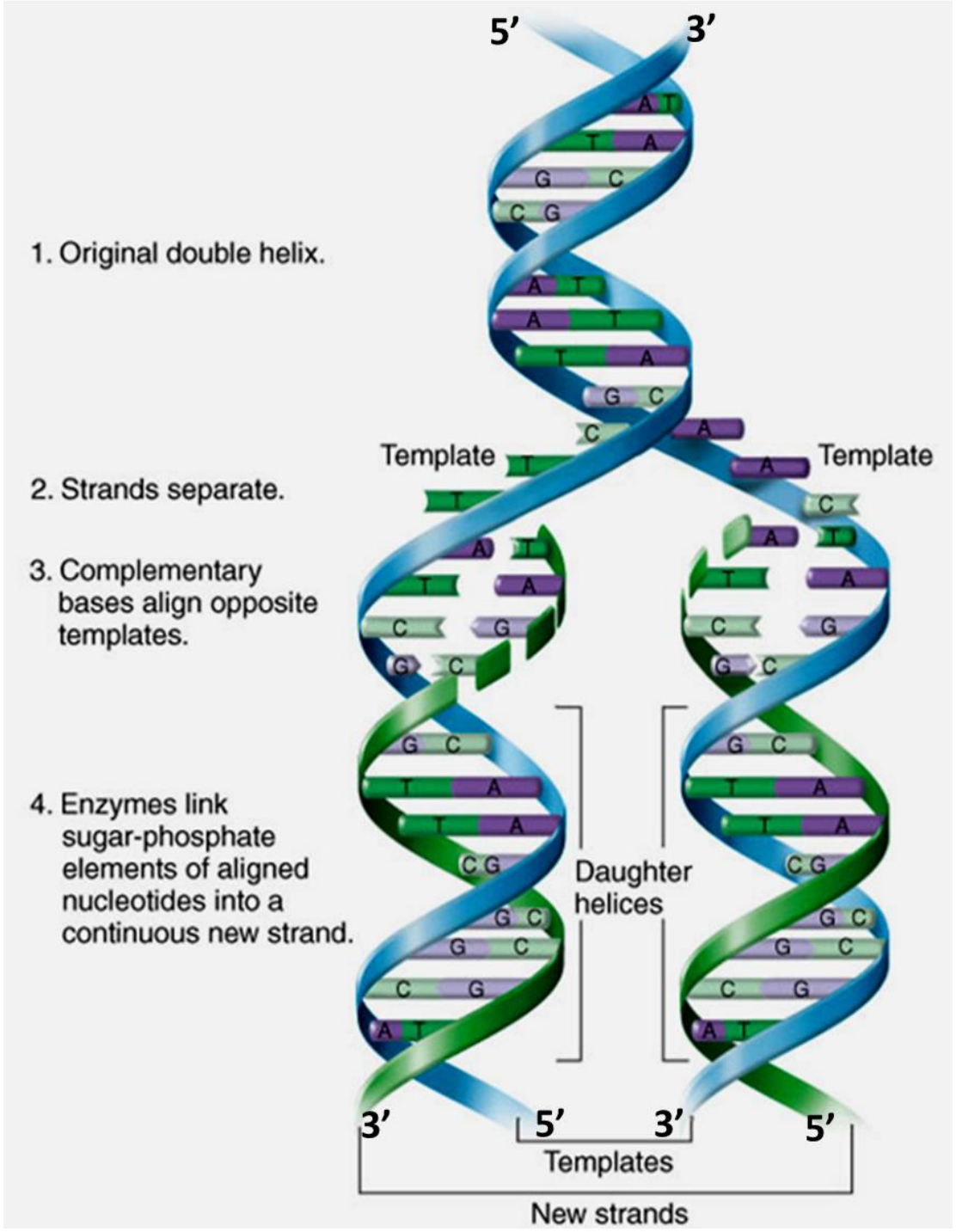
- Two identical new copies of the DNA double helix are produced during replication
- Each new strand is complementary to its old template strand
- In each new helix, one strand is the old template and the other is newly synthesized.



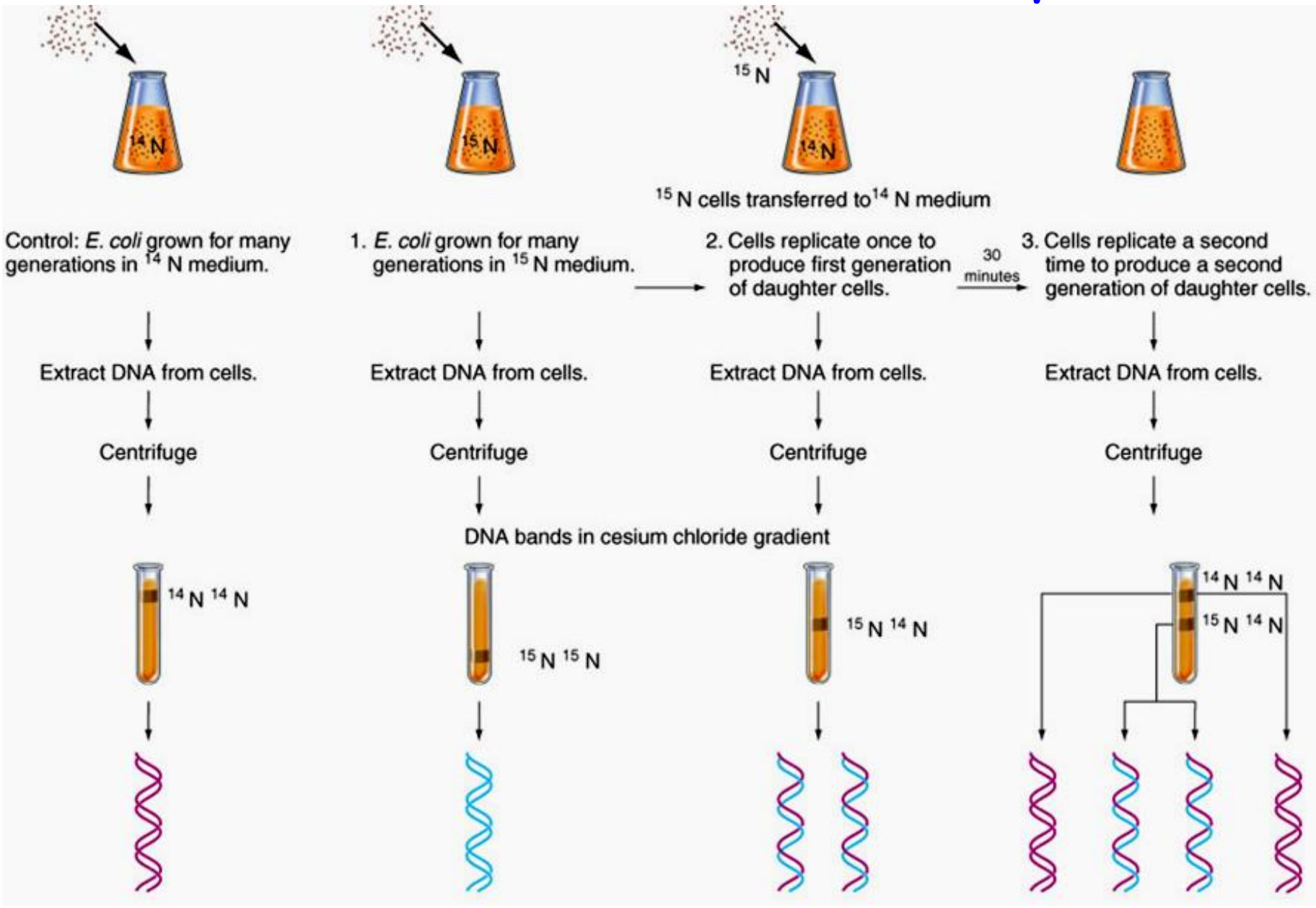
Semi-conservative replication:

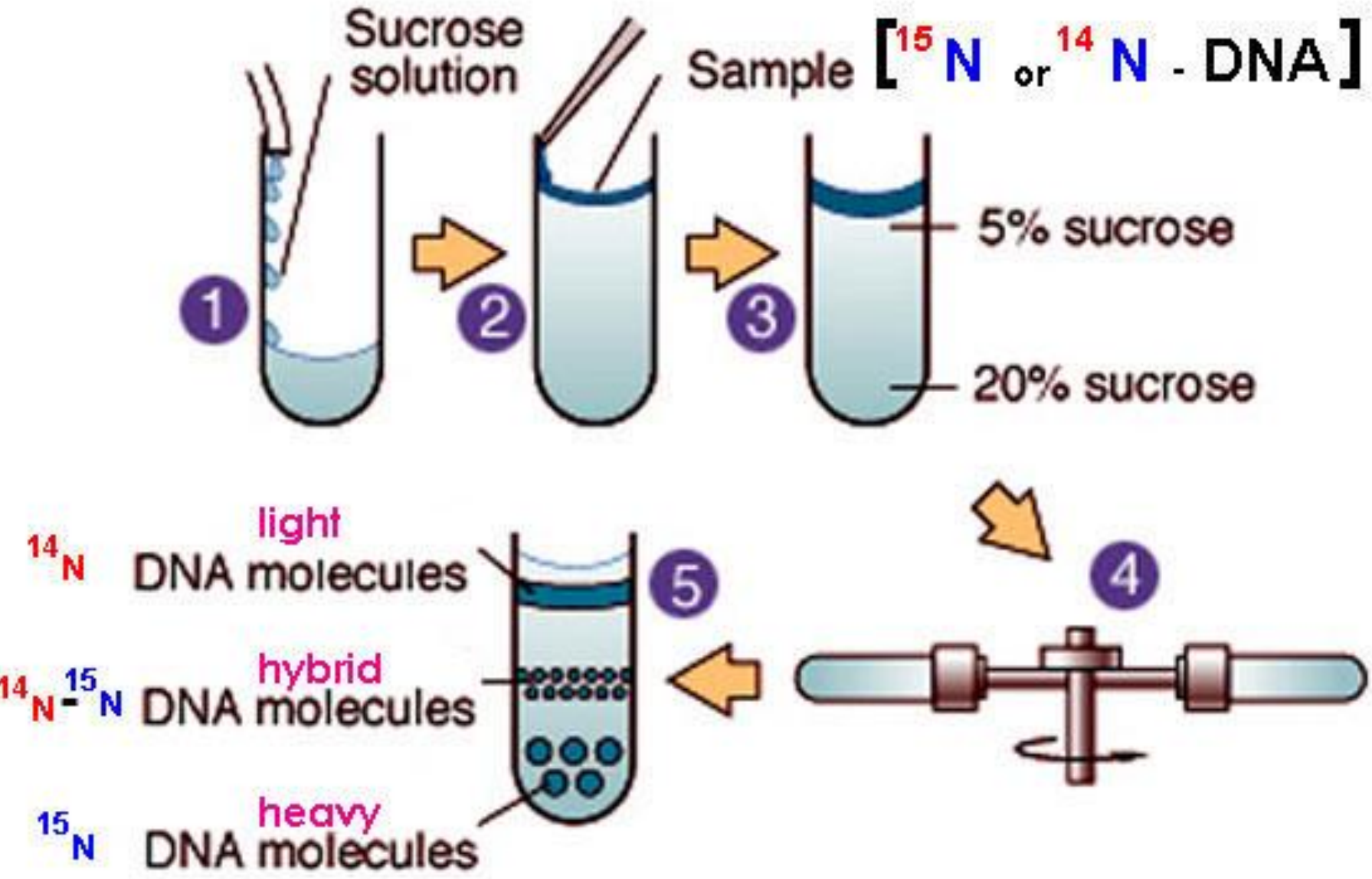
One strand of duplex passed on unchanged to each of the daughter cells.

This 'conserved' strand acts as a template for the synthesis of a new, complementary strand by the enzyme DNA polymerase



How do we know that DNA replication is semiconservative? - Meselson-Stahl experiments




Sucrose solution
Sample [15N or 14N - DNA]
1
2
3
5% sucrose
20% sucrose
4
light
14N DNA molecules
hybrid
14N-15N DNA molecules
heavy
15N DNA molecules

Meselson and Stahl's Experiment

- **Two isotopes of nitrogen:**
  - ^{14}N common form; **^{15}N** rare heavy form
  - *E. coli* were grown in a **^{15}N** media first, then transferred to **^{14}N** media.
  - Cultured *E. coli* were subjected to equilibrium density gradient centrifugation.

DNA Replication

- Replication = DNA copies itself exactly
  - ✓**(Occurs within the nucleus)**
- Any mistake in copying = mutation
- DNA mutation = chromosomal mutation

A. Basic Facts of DNA Replication

1. Complementary base pairing

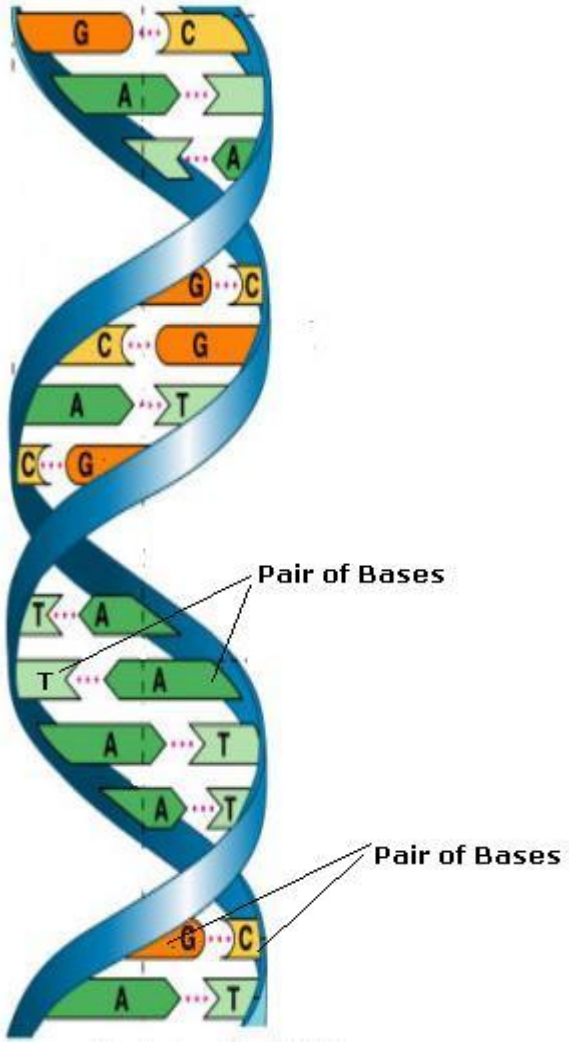
makes replication possible

C - G

A - T

A. Basic Facts of DNA Replication

2. One side of DNA molecule is a ***template*** for making the other side (strand)



B. Process of DNA Replication

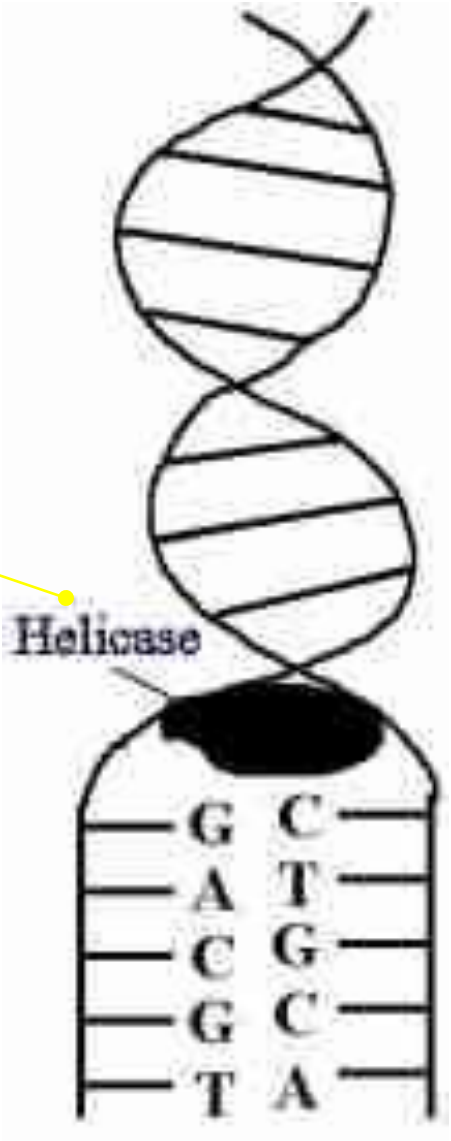
1. Uncoil & unzip DNA molecule

- **Enzyme (-ase)**

  breaks *weak*

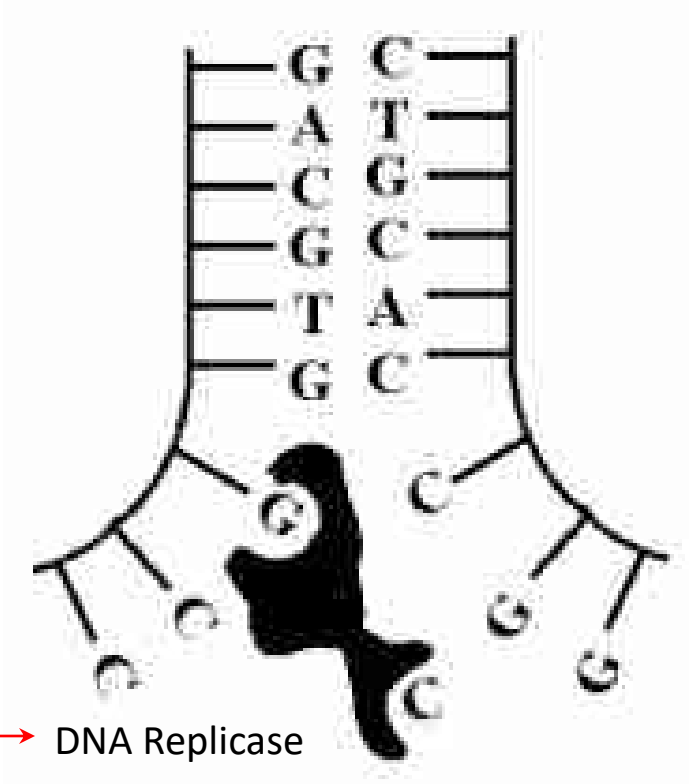
  Hydrogen Bonds

  between bases



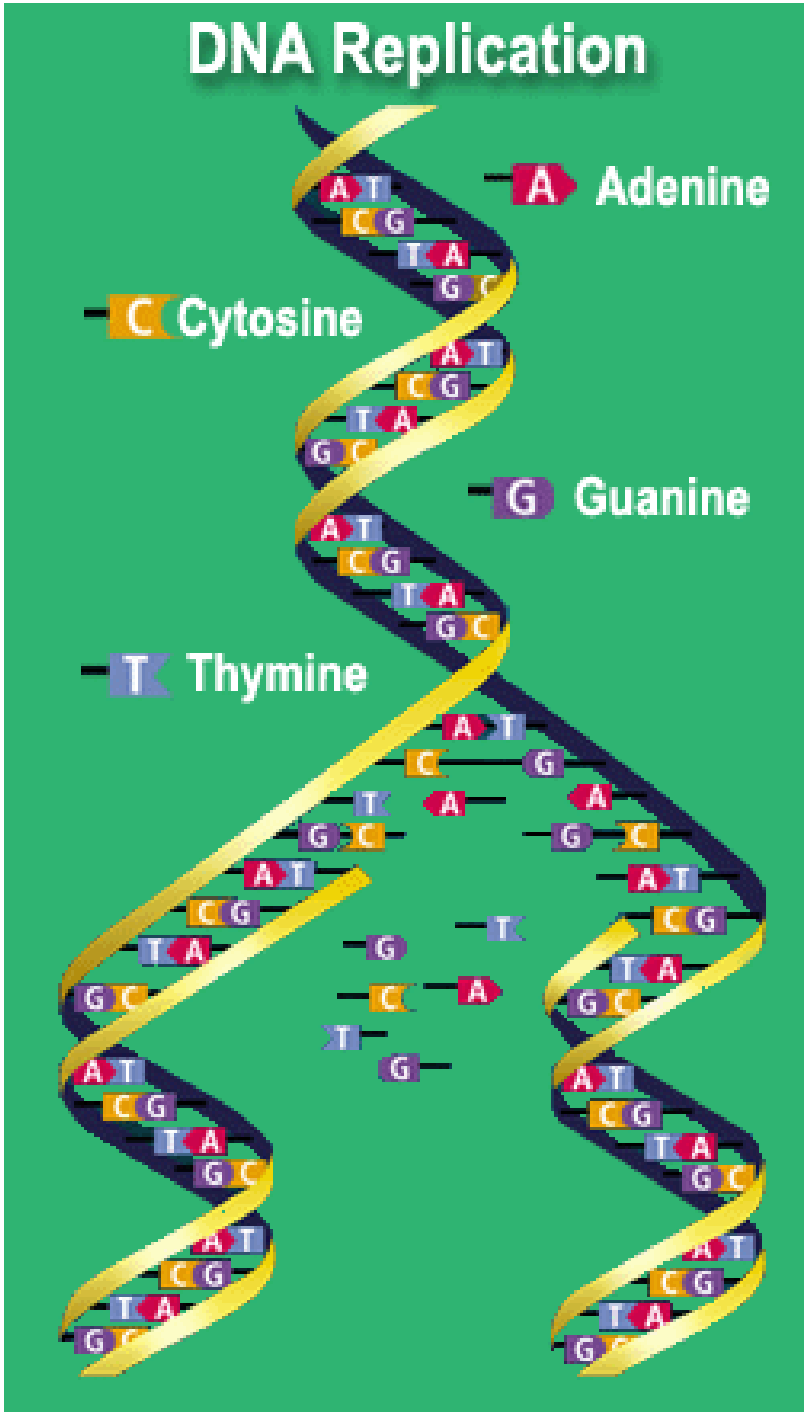
B. Process of DNA Replication

2. Enzyme brings in complementary N-bases



B. Process of DNA Replication

3. Insert N-bases



Replication

C - G

A - T

DNA vs. RNA

DNA	**RNA**
Sugar = deoxyribose	Sugar = ribose
Double-stranded molecule	Single-stranded molecule
Thymine bonds with adenine	Uracil instead of thymine

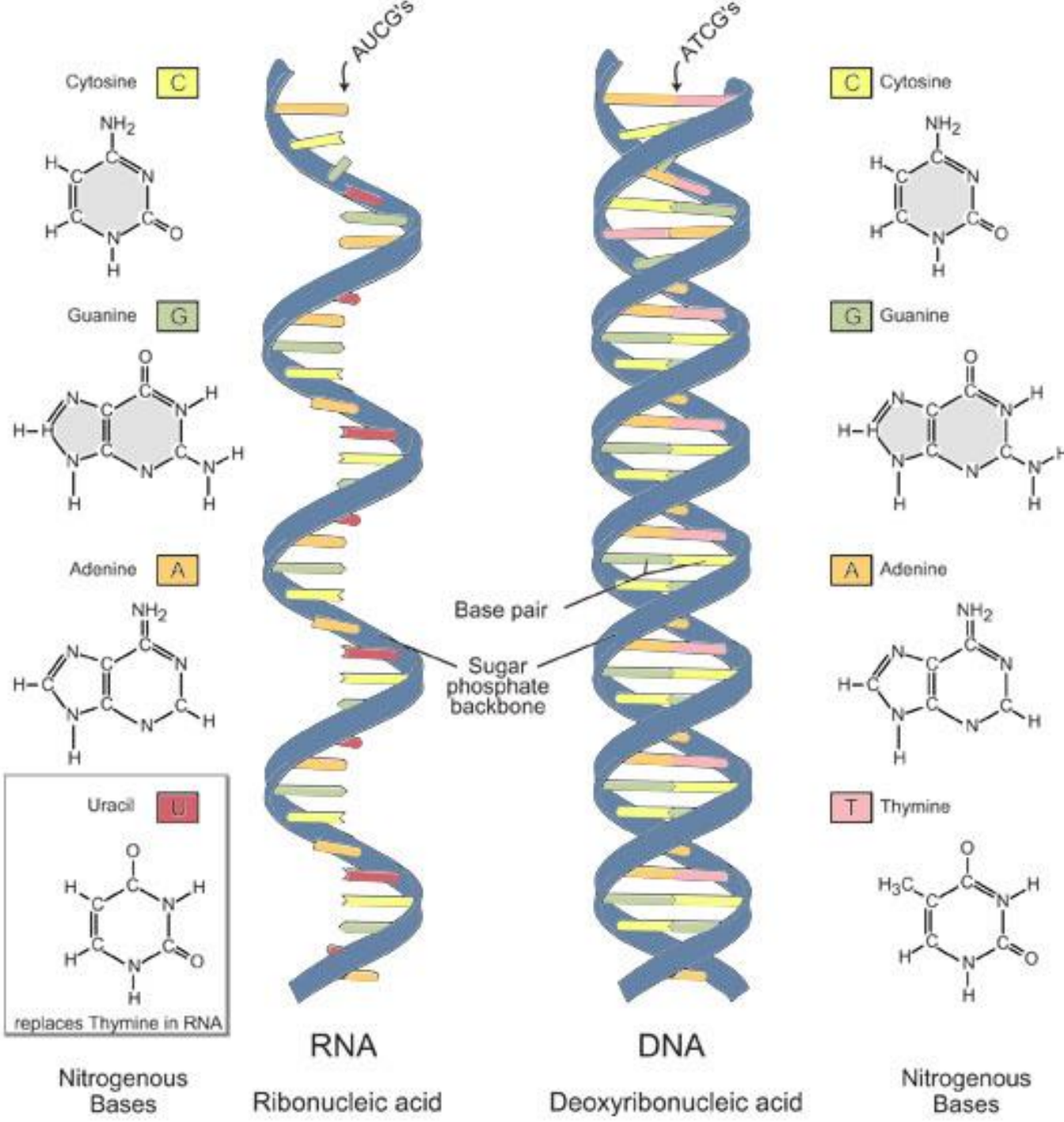
DNA vs. RNA

DNA	RNA
Nuclear DNA Mitochondrial DNA Chloroplast DNA Plasmid DNA	mRNA = messenger tRNA = transfer rRNA = ribosomal
Nuclear DNA never leaves the nucleus	Assembled in nucleus, moves to cytoplasm (leaves the nucleus)

DNA

vs.

RNA


AUCG's
ATCG's
Cytosine C
Guanine G
Adenine A
Uracil U
replaces Thymine in RNA
Base pair
Sugar phosphate backbone
RNA
DNA
Nitrogenous Bases
Ribonucleic acid
Deoxyribonucleic acid
C Cytosine
G Guanine
A Adenine
T Thymine
Nitrogenous Bases

DNA Replication Steps

- ***Initiation***
  - involves assembly of replication fork (bubble) at origin of replication
    - sequence of DNA found at a specific site
- ***Elongation***
  - Parental strands unwind and daughter strands are synthesized.
  - the addition of bases by proteins
- ***Termination*:**
  - the duplicated chromosomes separate from each other. Now, there are 2 IDENTICAL copies of DNA.

Polymerases & polymerizing reaction

- Sir Arthur Kornberg (1957) discovered DNA polymerase I in *E. coli.*
- DNA polymerase I or DNA Pol I or Pol I or ***Kornberg polymerase*** has a molecular weight of 109KD.
- Later pol II, pol III, pol IV and pol V were discovered.
- DNA pol III is more efficient & has high processivity than pol I and is the major replicating enzyme in *E. coli.*
- The primary function of all polymerases is to polymerize and they polymerize in 5' → 3' using nucleoside triphosphates as monomers.
- ***Polynucleoside phosphorylase** uses **Nucleoside diphosphates** rather than **NTPs for polymerization.**

Polymerases & polymerizing reaction

- **DNA polymerase I or DNA Pol I or Pol I or *Kornberg's* enzyme:-**
- DNA pol I has zinc in its structure and requires Magnesium for its activity.
- It has a molecular weight of 109KD.
- When DNA pol I treated with subtilisin it will be divided in to 2 fragments.
- The large fragment is known as Klenow fragment.
- No specific name was given to small fragment.

- **Properties of DNA pol I** are

(1) 5' → 3' polymerization

(2) 3' → 5' exonuclease activity / editing function / proof reading function

(3) 5' → 3' exonuclease

(4) Nick translation

(5) Strand displacement

(6) Endonuclease

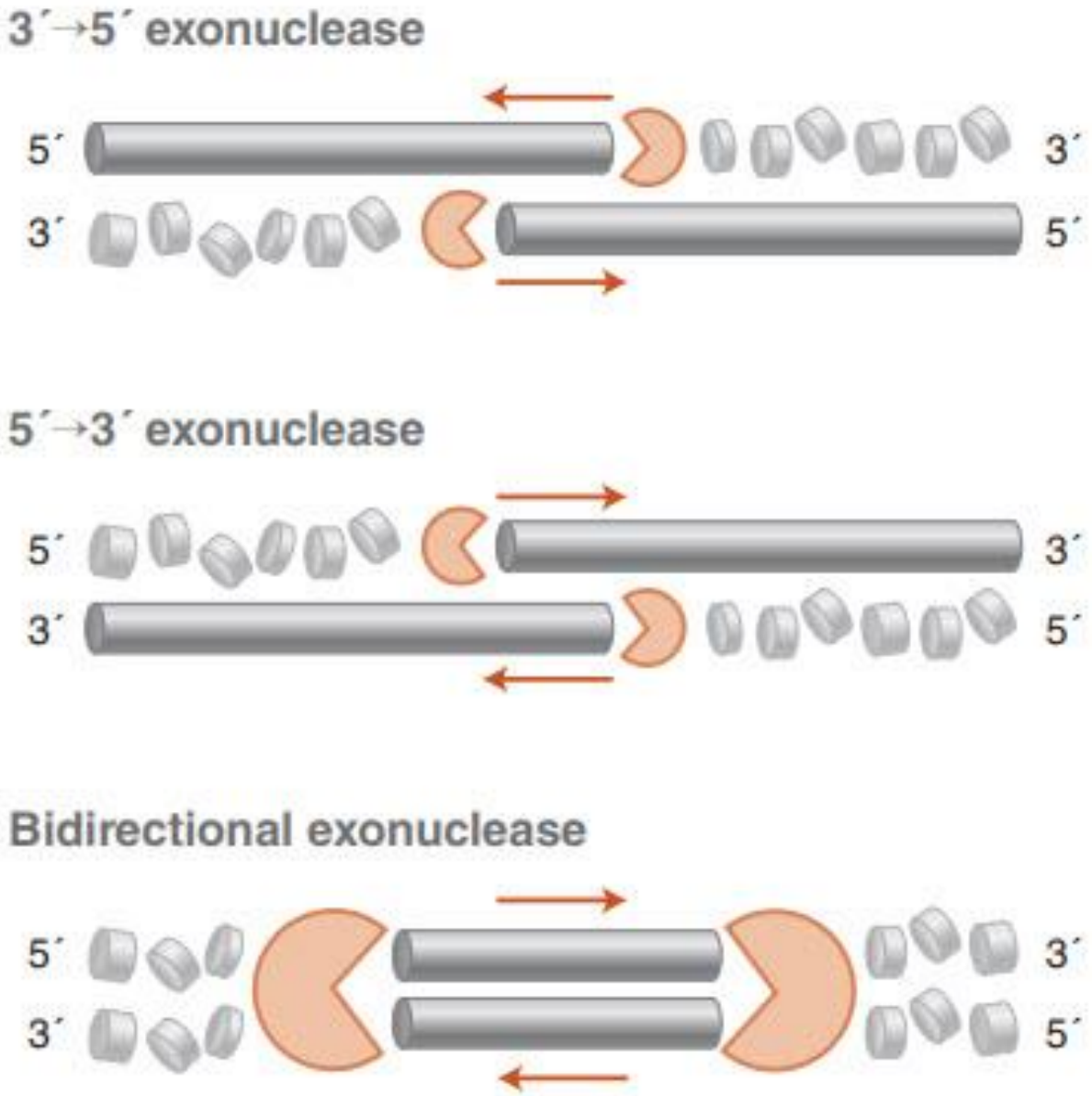
Properties of DNA Pol I

- DNA polymerase cannot initiate DNA synthesis by itself.
- It can add a nucleoside triphosphate only to the 3’ OH group of an existing oligonucleotide.
- The incoming NTP joins with OH group of the existing nucleotide and forms a phosphodiester bond and releases a Pyrophosphate.
- This pyrophosphate will be readily converted into in inorganic phosphate by pyrophosphatase.
- If the pyrophosphate level accumulates DNA polymerase catalyzes the depolymerization of DNA rather than polymerization.

Primer

- DNA polymerase cannot initiate DNA synthesis by itself.
- A primer will be synthesized by RNA polymerase (different from normal RNA polymerase that transcribes).
- Primer is a short oligonucleotide (10-30 nucleotides) made up of RNA/ DNA/ proteins and provides the 3'OH group required for extension by polymerase.
- Usually primer is complementary to 3' end of the template.

EXONUCLEASES



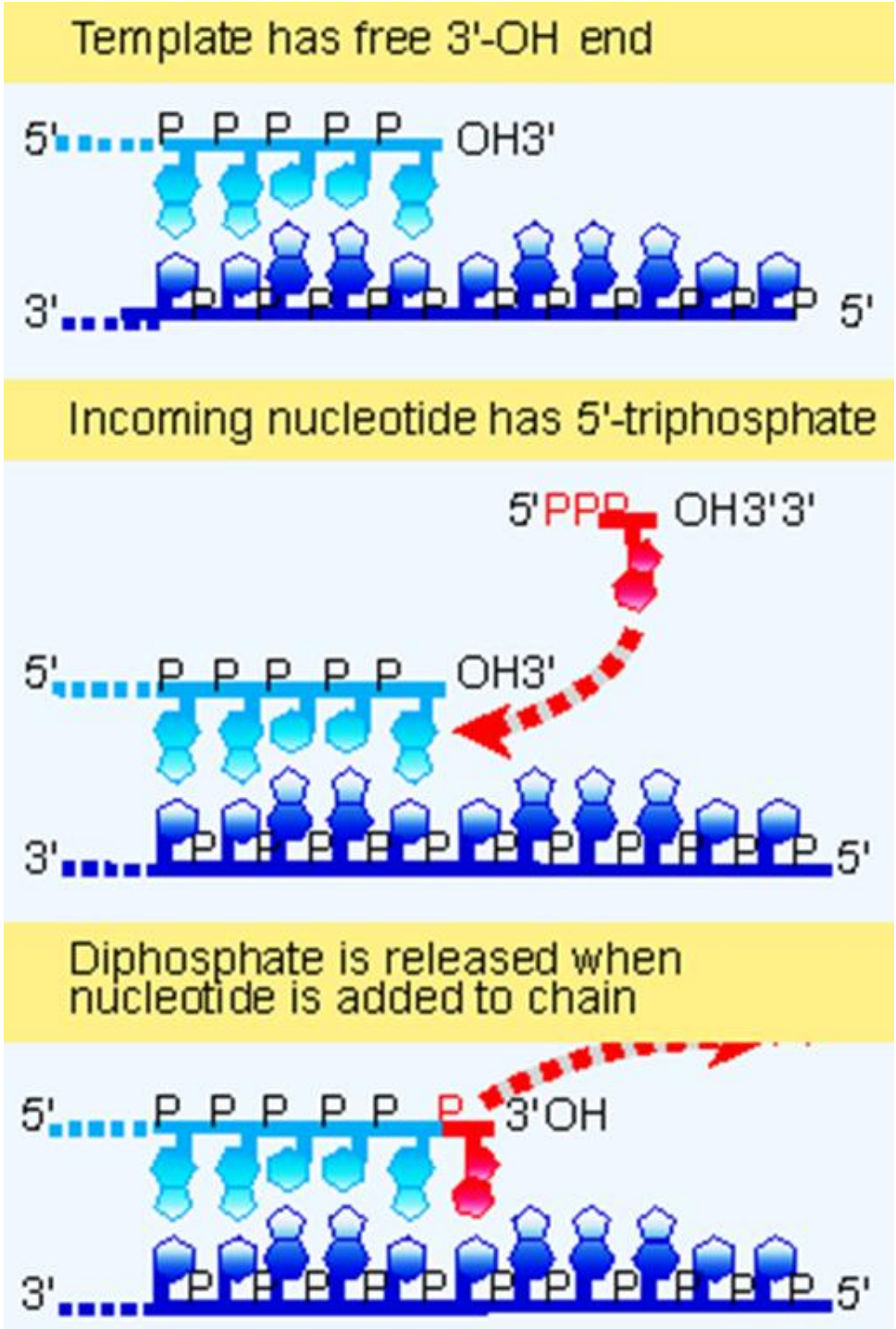
5' → 3' polymerization:

To exhibit this property DNA polymerase **requires**

- A single stranded DNA which provides information that is to be copied – the **template**
- 4 types of **Nucleoside triphosphates**.
- **Salts** and accessory requirement.
- **Primer** hydrogen bonded to the **3' terminus** of the template.

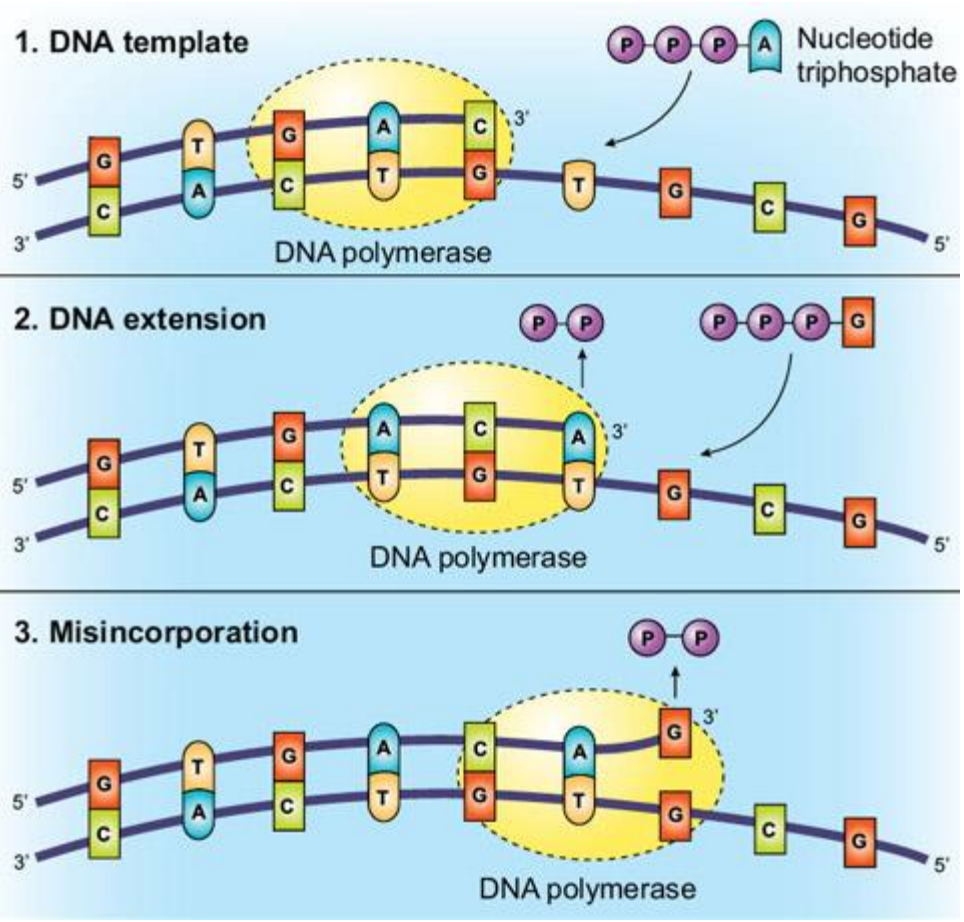
Properties of DNA Pol I

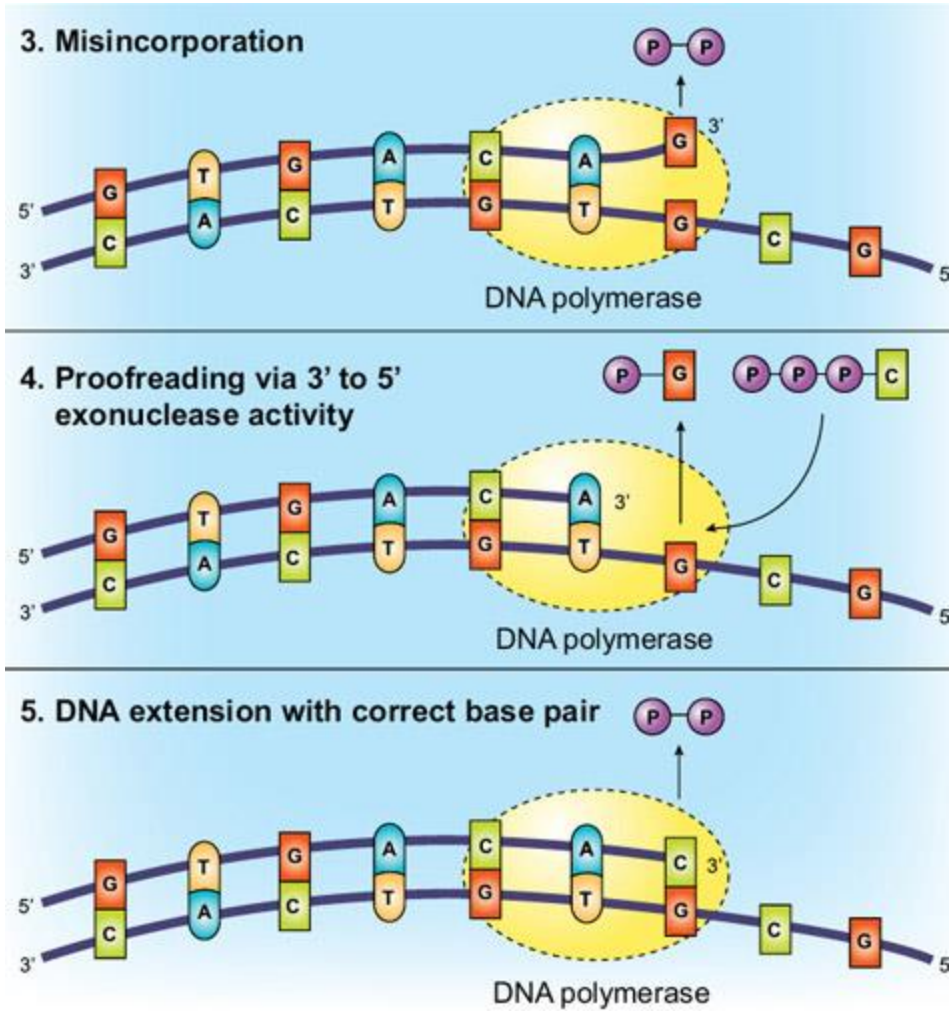
1) 5-3’ polymerization

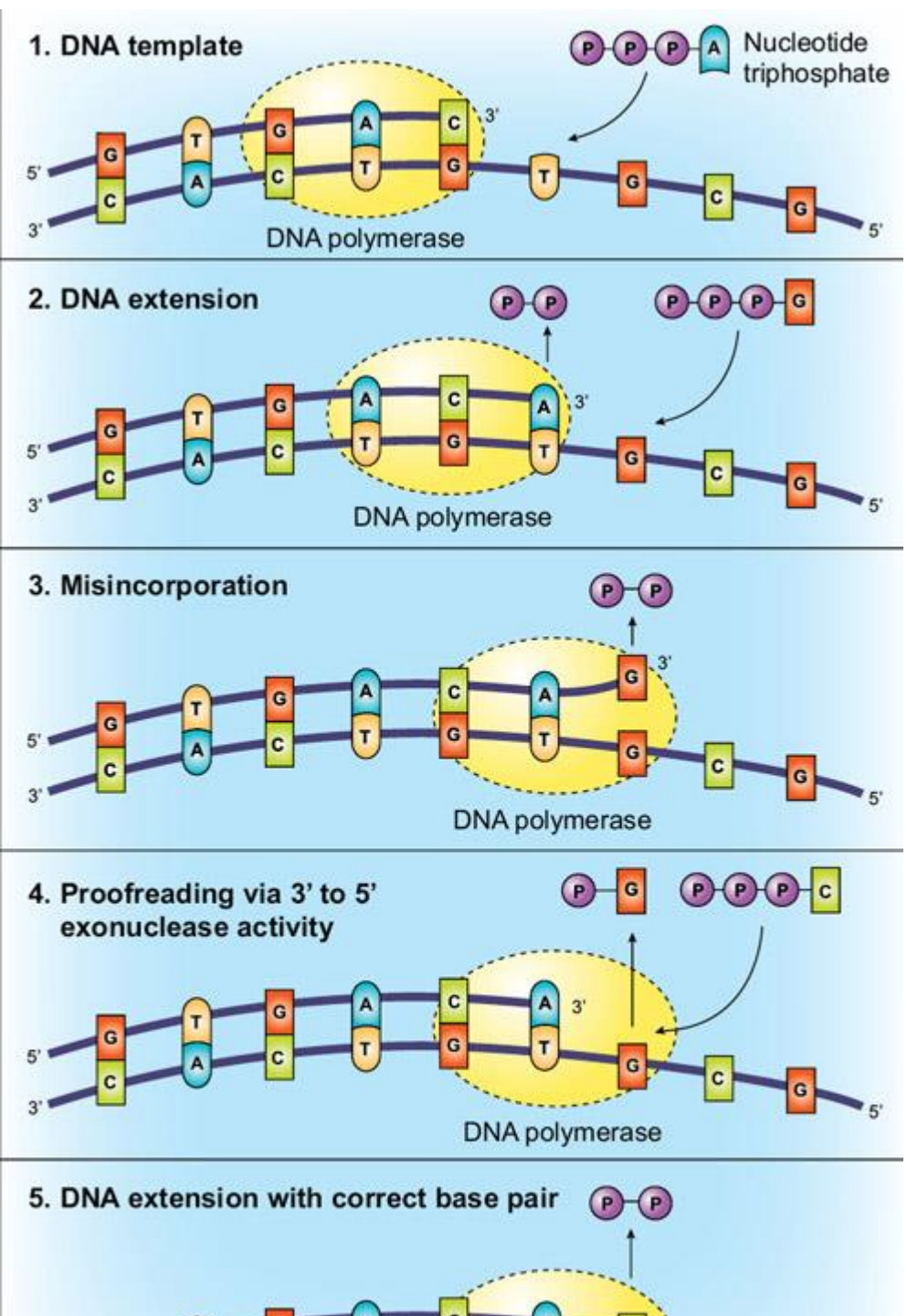


Properties of DNA Pol I

2) 3-5’ Exonuclease activity/ Editing function/ Proof reading function







Properties of DNA Pol I

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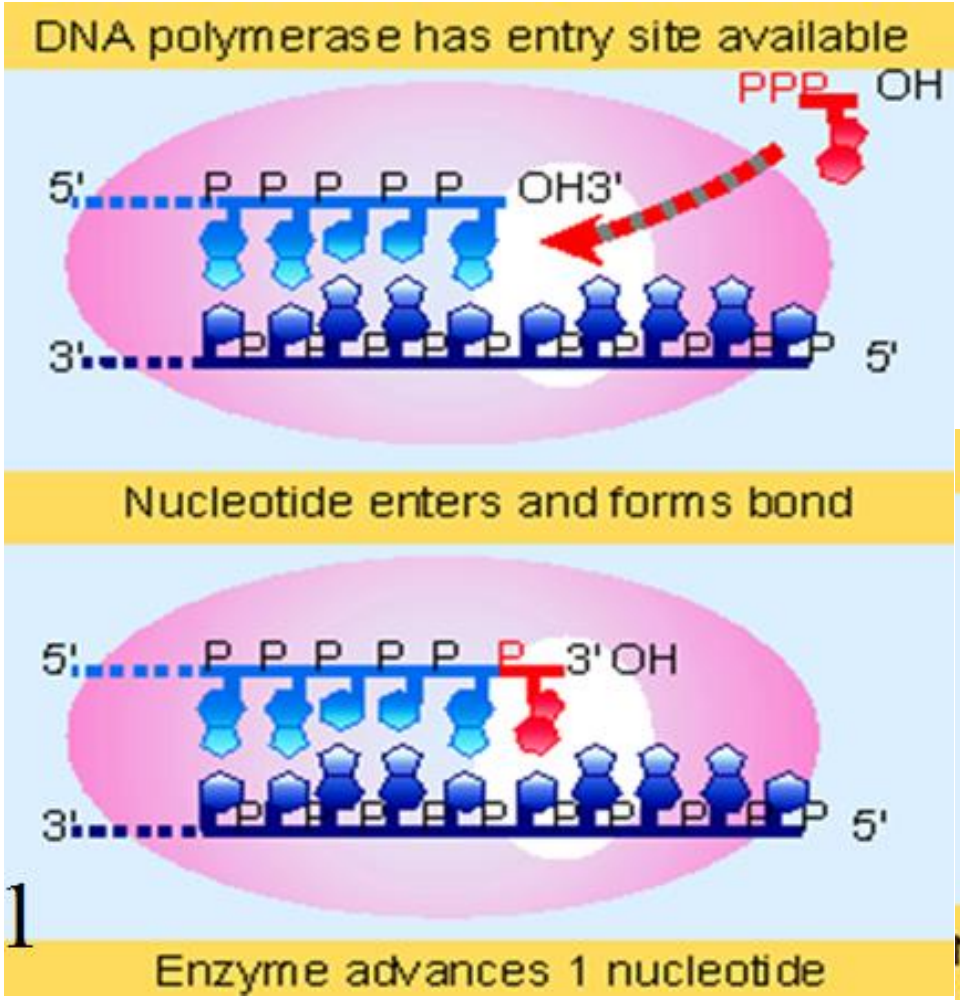

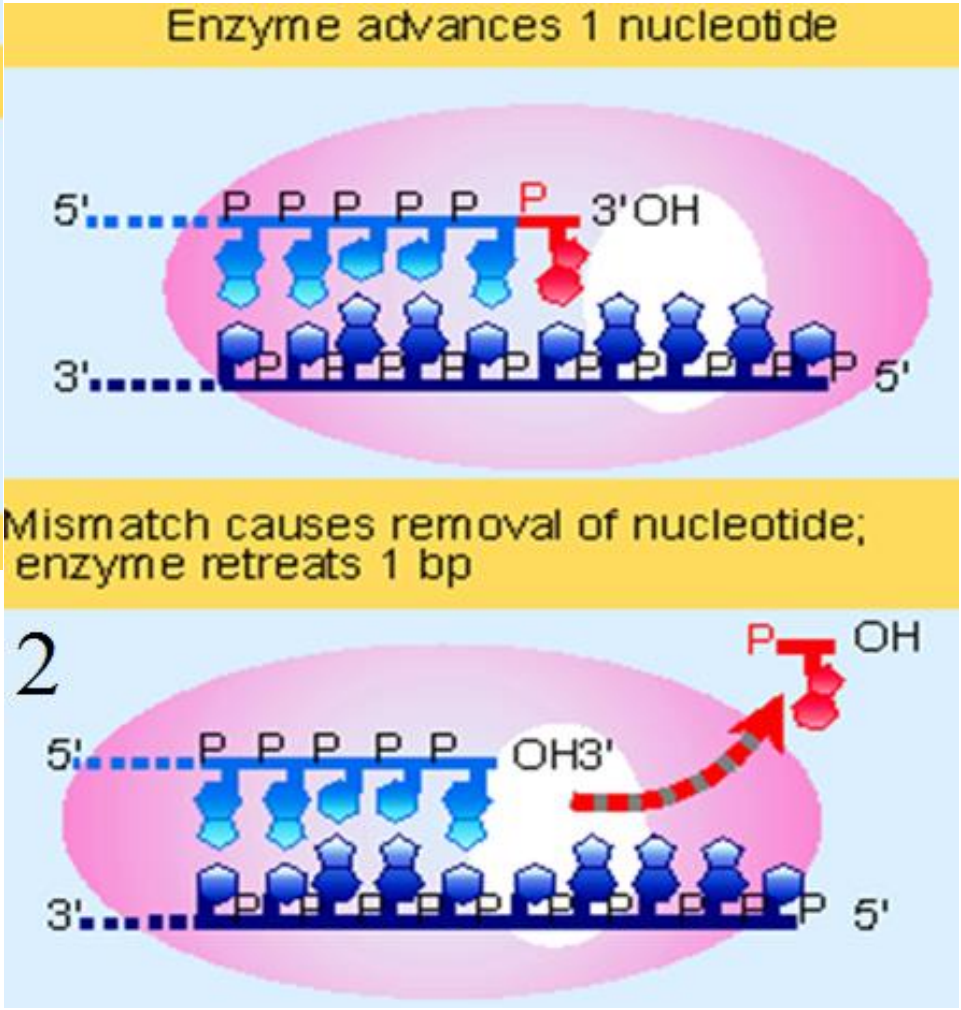

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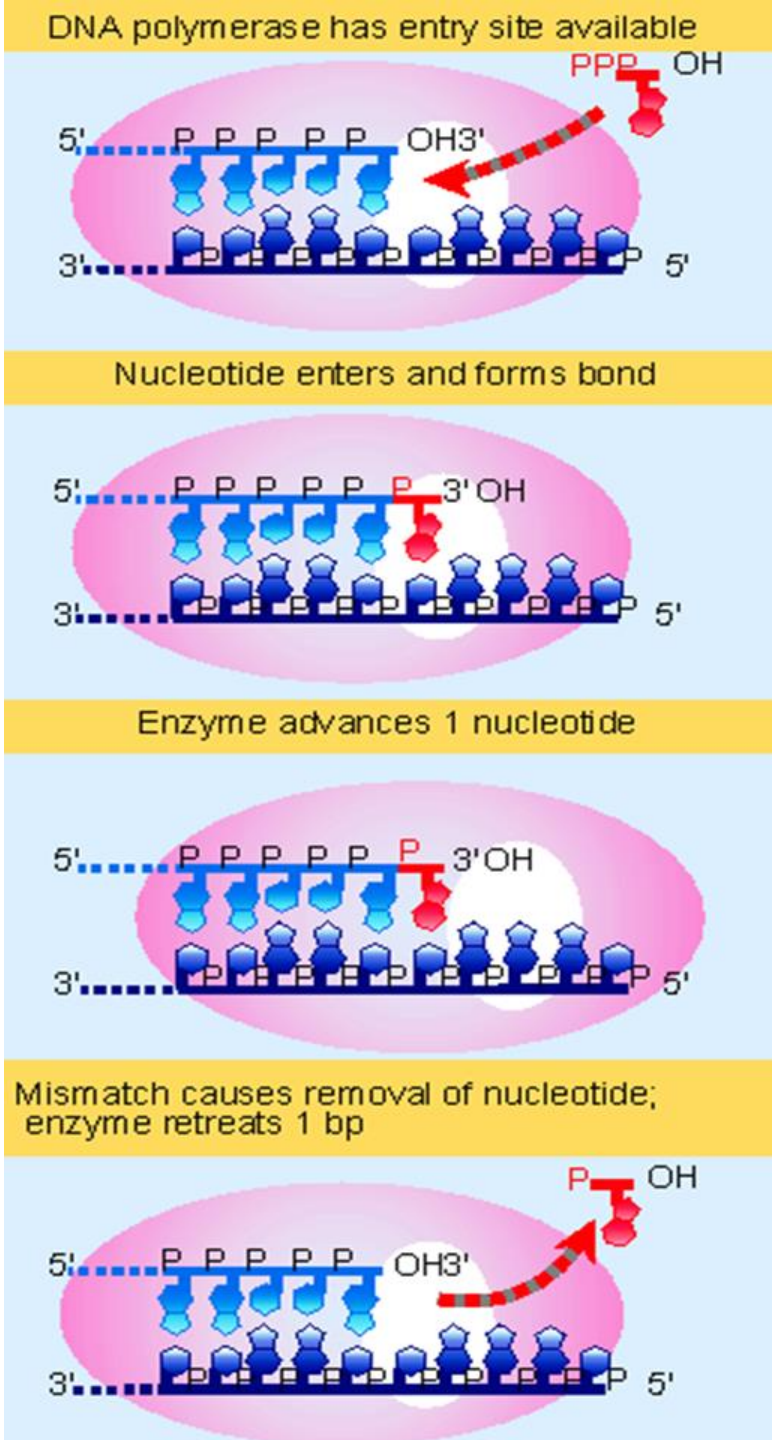


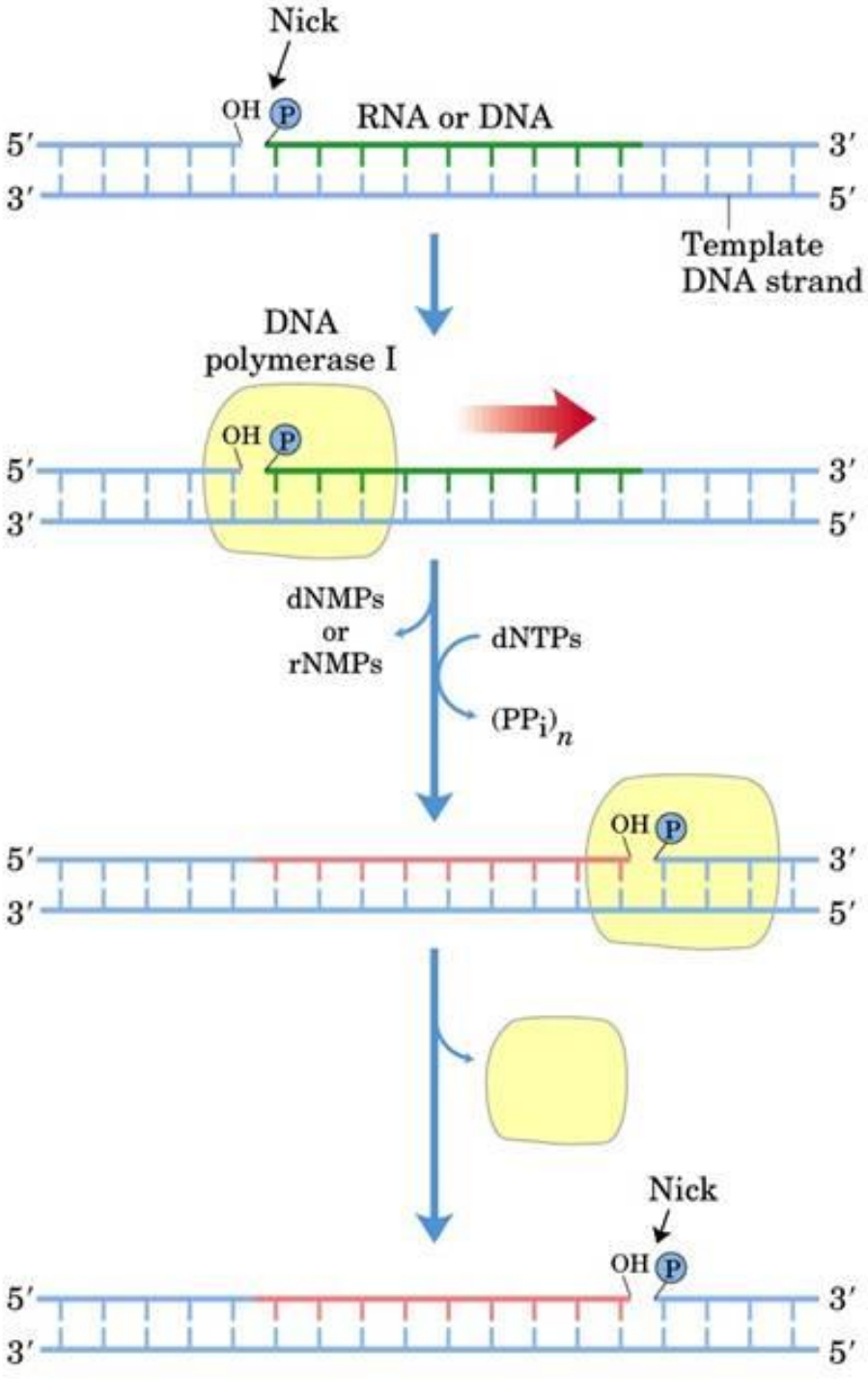
Properties of DNA Pol I

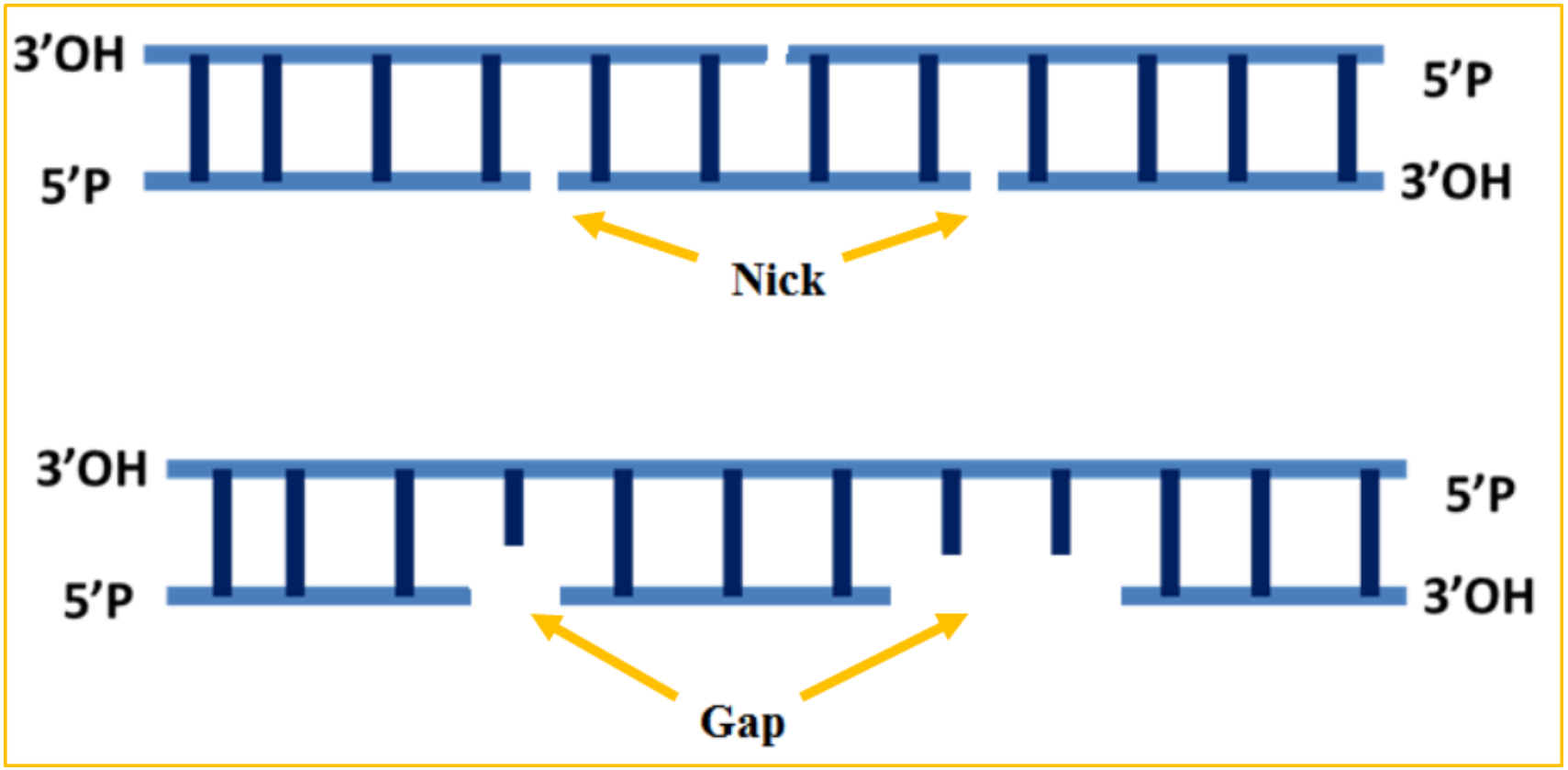
- **5' → 3' exonuclease activity:**
- DNA polymerase I exhibits this property only under certain conditions

1. DNA polymerase deletes the nucleotide starting only from the 5’ Phosphate end. If the conditions are favorable then it can remove more than one nucleotide.
2. It can remove only hydrogen bonded or base paired nucleotides.
3. It can act either at a nick or a gap as long as the nucleotide present at 5’ end is hydrogen bonded to the complimentary base and has a phosphate terminus.
4. It cannot act on ssDNA.
5. It cannot delete modified bases or protected bases.
6. It cannot act if the 5’ terminus is dephosphorylated.

- *The main function of 5' → 3' Exonuclease activity of DNA pol I is to replace the primer (made of RNA) with DNA. It is also helpful in DNA repair mechanisms.

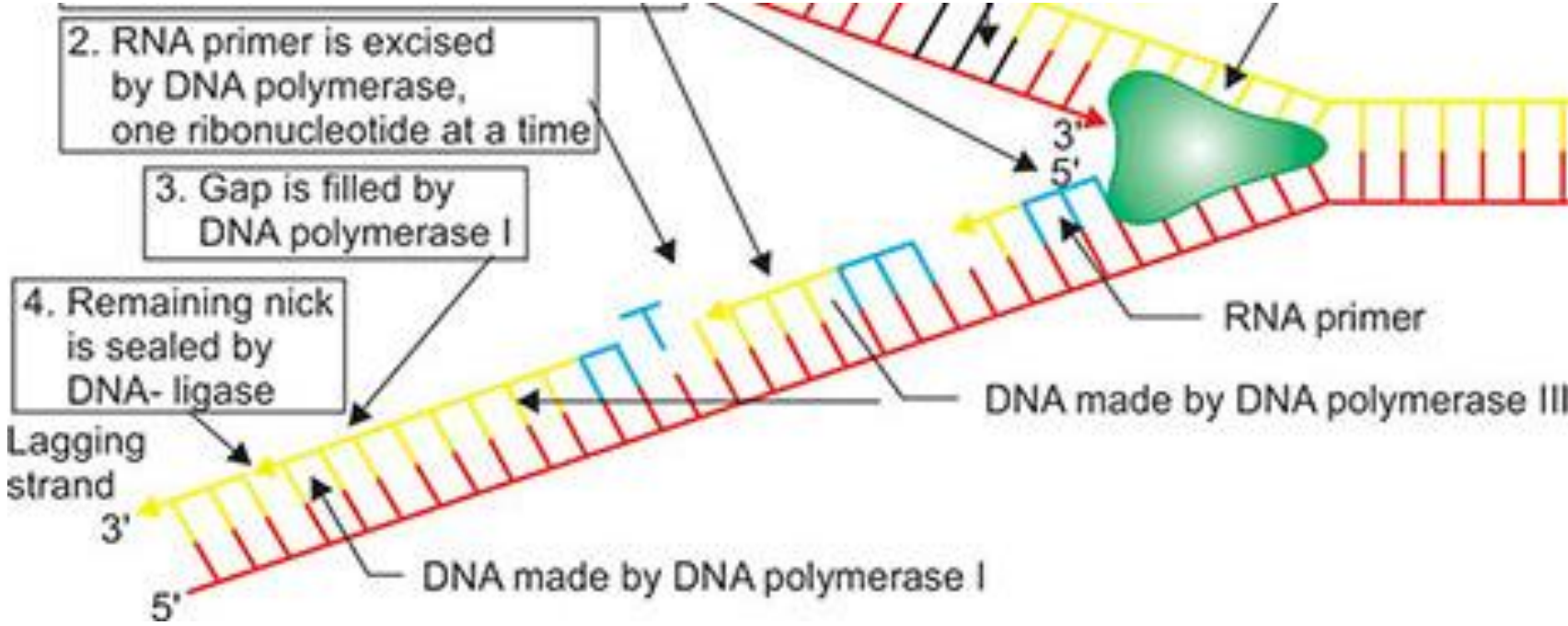
- **Polymerase I** has a 5`→ 3` exonuclease activity in order to remove the RNA primer. In addition to that, it also possesses 3`→ 5` exonuclease activity which aims at proofreading the DNA sequence that has replaced the RNA primer.



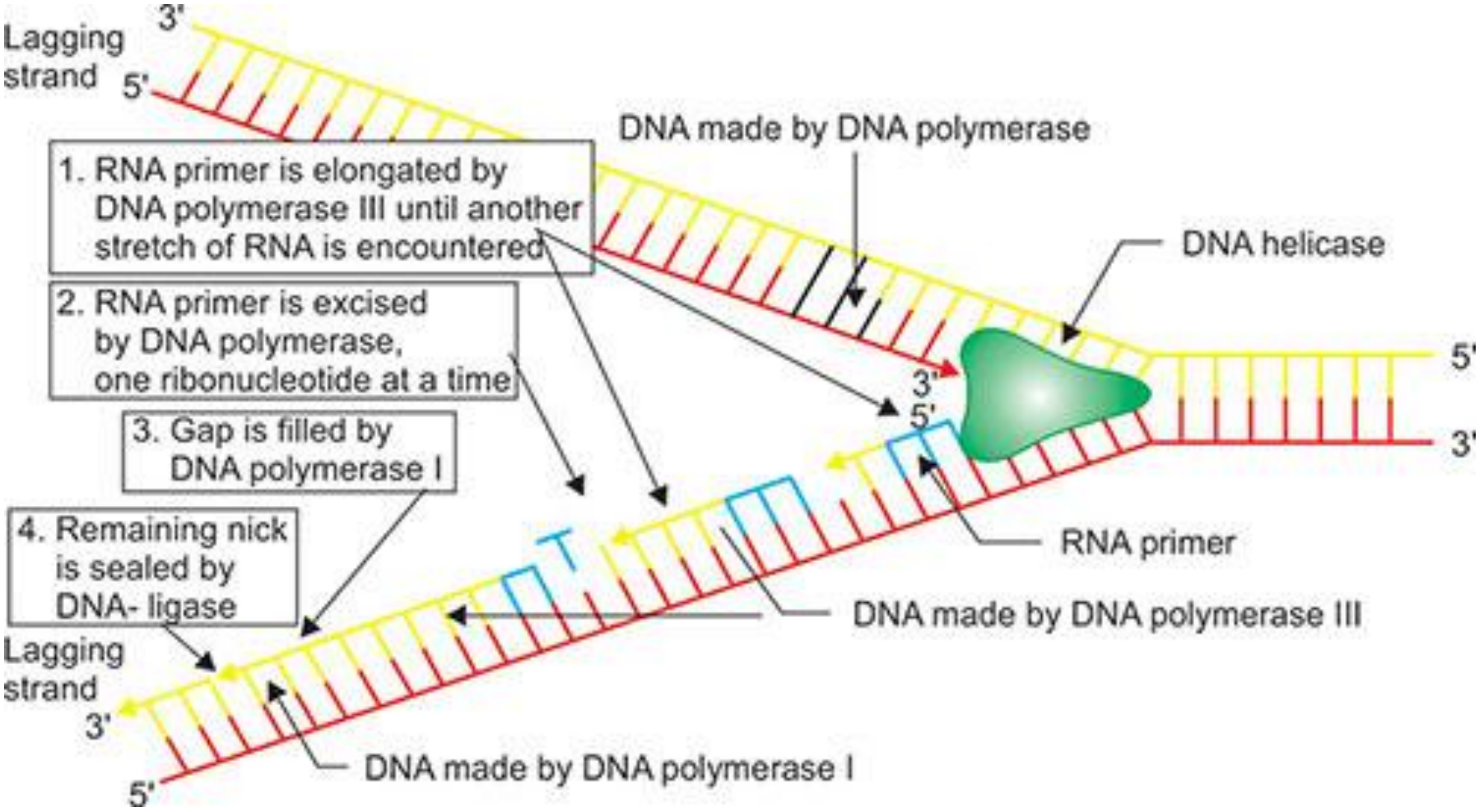


Nick: **Breakage** of **Phosphodiester** bond between **adjacent** nucleotides.

Gap: **At least** one **nucleotide** is **missing** in one of the 2 strands, hence at least at **one** location DNA is **single** stranded



Replicating DNA has Nicks and Gaps

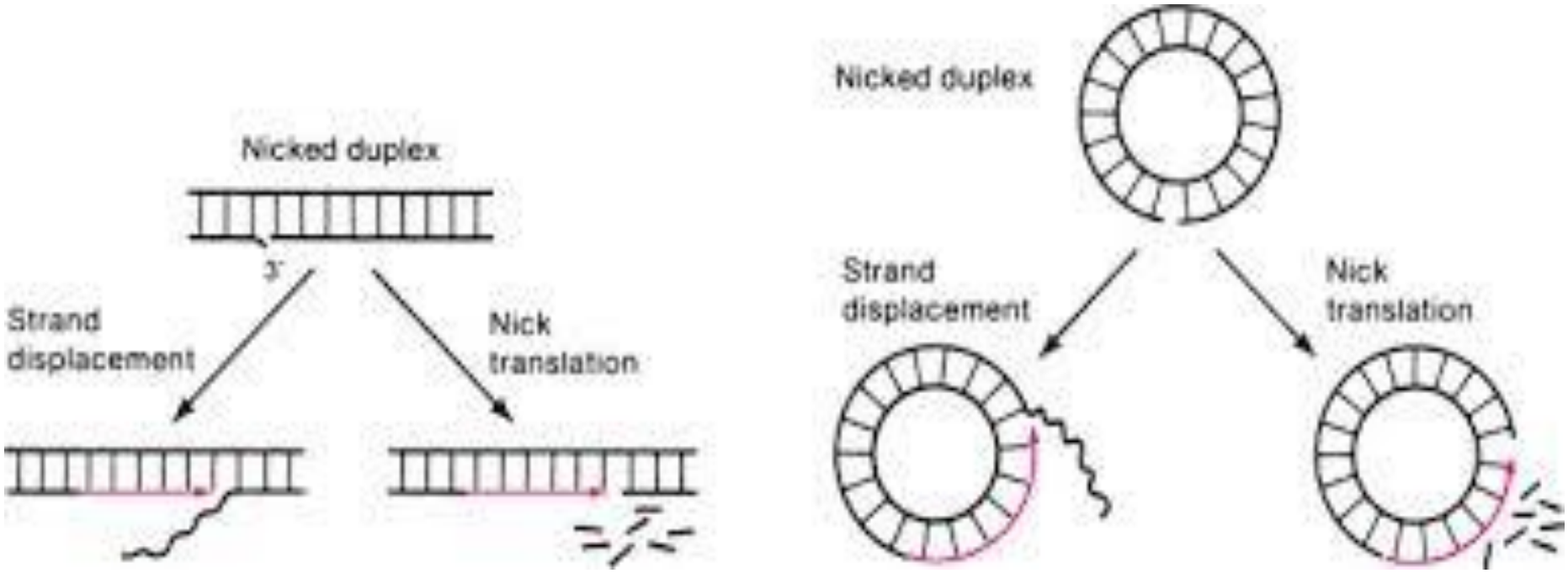


Replicating DNA has Nicks and Gaps

Properties of DNA Pol I

- DNA polymerase cannot initiate DNA synthesis by itself. It can add a nucleoside triphosphate only to the 3’ OH group of an existing oligonucleotide.
- The incoming NTP joins with OH group of the existing nucleotide and forms a phosphodiester bond and releases a Pyrophosphate.
- This pyrophosphate will be reading converted into in inorganic phosphate by pyrophosphatase.
- If the pyrophosphate level accumulates DNA polymerase catalyzes the depolymerization of DNA rather than polymerization.

Properties of DNA Pol I



4) Unaided Nick Translation by DNA polymerase 1

Properties of DNA Pol III

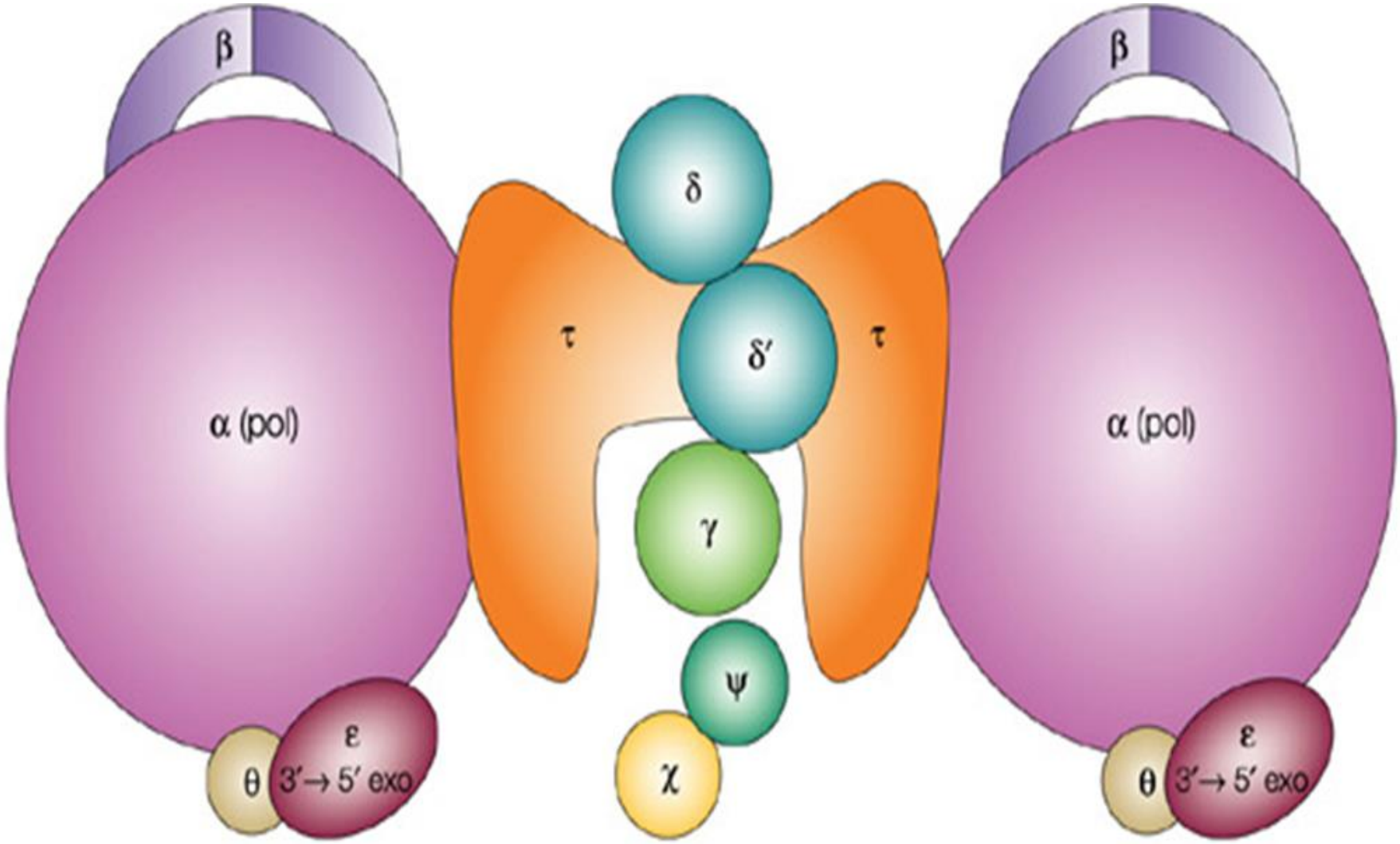
- DNA pol III is much more complex enzyme than DNA pol I and DNA pol II.
- **Holoenzyme** is a general name for a multiprotein complex in which a **core enzyme** activity is associated with additional components that enhance function.

Properties of DNA Pol III

- **DNA Pol III** holoenzyme comprises of at least 10 enzymatic subunits. The subunits are

1) **α** (5' → 3' polymerization),
2) **β** (DNA clamp required for optimal processivity),
3) **ε** (3' → 5' exonuclease activity),
4) **θ** (stimulates editing function),
5) **τ** (dimerization)
6) **γ (7) δ (8) δ′(9) χ (10) ψ**.

Properties of DNA Pol III



Properties of DNA Pol III

- Polymerization activity is seen with α subunit and editing with ε subunit.
- These two subunits along with θ unit form a core enzyme which polymerizes DNA, with a low **processivity.**

Properties of DNA Pol III

- Two core polymerases are linked together by a dimmer of **τ** subunits.
- This dimeric polymerase then complexes with a single clamp loading complex (γ complex) which consists of 6 subunits of 5 types, γ_2, δ, δ′, χ, ψ.
- **γ δ δ′ χ ψ complex**→ clamp loading complex that loads β subunits on core enzyme associated with lagging strand synthesis at the beginning of each Okazaki fragments.
- This enzyme's processivity is enhanced by two β clamps, each formed by 2 identical subunits of β. Thus a heterodimer of pol III holoenzyme is formed.

Properties of DNA Pol III

- The presence of γ complex allows β clamp to dissociate from the template when it encounters a primer or 5’ end of Okazaki fragments and associate when the enzyme is loaded at the next available template.
- This DNA pol III exhibits polymerization in 5' → 3' direction and proof reading function similar to pol I but with a greater speed.
- The DNA pol III also exhibits 5' → 3' exonuclease activity only on ssDNA and hence it cannot act at a nick.

Properties of DNA Pol III

- The precise biological role of this 5' → 3' exonuclease activity of pol III is not known. It doesn't have Endonuclease activity.
- To exhibit strand displacement it requires additional proteins and ATP to unwind the DNA.
- DNA pol III cannot carry out strand displacement on its own.

DNA Pol II, IV & V

- These enzymes are useful in DNA repair mechanisms

Replication-Requirements

- **Replisome:** Replication is a complicated process and requires at least 20 different enzymes or proteins. This complex of all enzymes or protein is known as DNA replicase system or replisome.
- Its components include proteins - DnaA, DnaB, DnaC, DnaG, HU (Histone like) protein, SSB protein, DNA Pol I, DNA Pol III, Topoisomerase, DNA ligase, Tus protein etc.

Replication-Requirements

- **Helicases:-**These helicases translocate on DNA in any one direction only.
- Based on their movement they are classified into 5'→3' helicases and 3'→5' helicases.
- Ex. DnaB protein exhibits 5' → 3' polarity useful in *E. coli* chromosomal replication. Rep protein 3' → 5' polarity useful in σ replication.
- In DNA molecules having topological constraints they (helicases) work together with a topoisomerase to relieve the strain caused due to torsion.

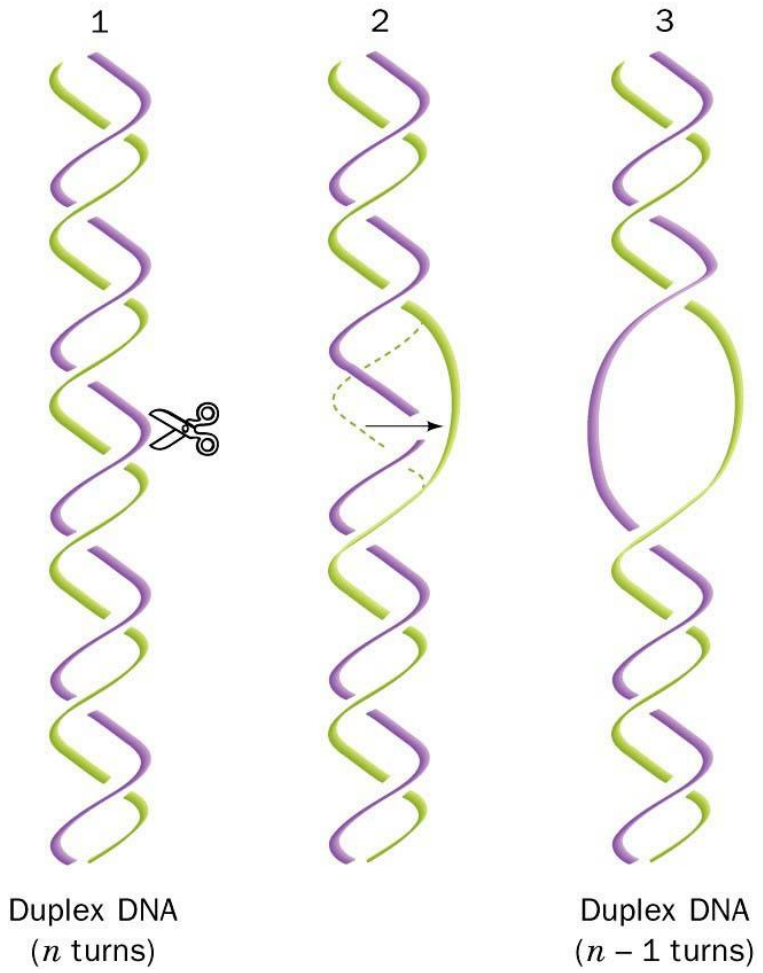
Replication-Requirements

- **Topoisomerases:-**
- These are the enzymes that catalyze the inter conversion of DNA topoisomers.
- They relax the torsional strain generated in DNA during replication due to the unwinding of DNA double helix by helicases.
- They also resolve catenae's and remove knots which arise during replication and recombination.
- Functionally topoisomerases have been classified into 2 classes' viz., class I topoisomerases and class II topoisomerases.

Replication-Requirements

- **Class I topoisomerases** relax the torsional strain by cleaving only one of the 2 strands followed by its ligation. Thus they can catenate or de-catenate only nicked DNA molecules. **They do not require any ATP.**
- **Mechanism:** These enzymes bind non-covalently to DNA. They cleave one strand and 5'-PO_4 group is covalently attached to tyrosine residue in the active site. Intact strand is passed through the break. Then the broken strand is joined. E.g. Topo I, Topo III, Topoisomerase I.

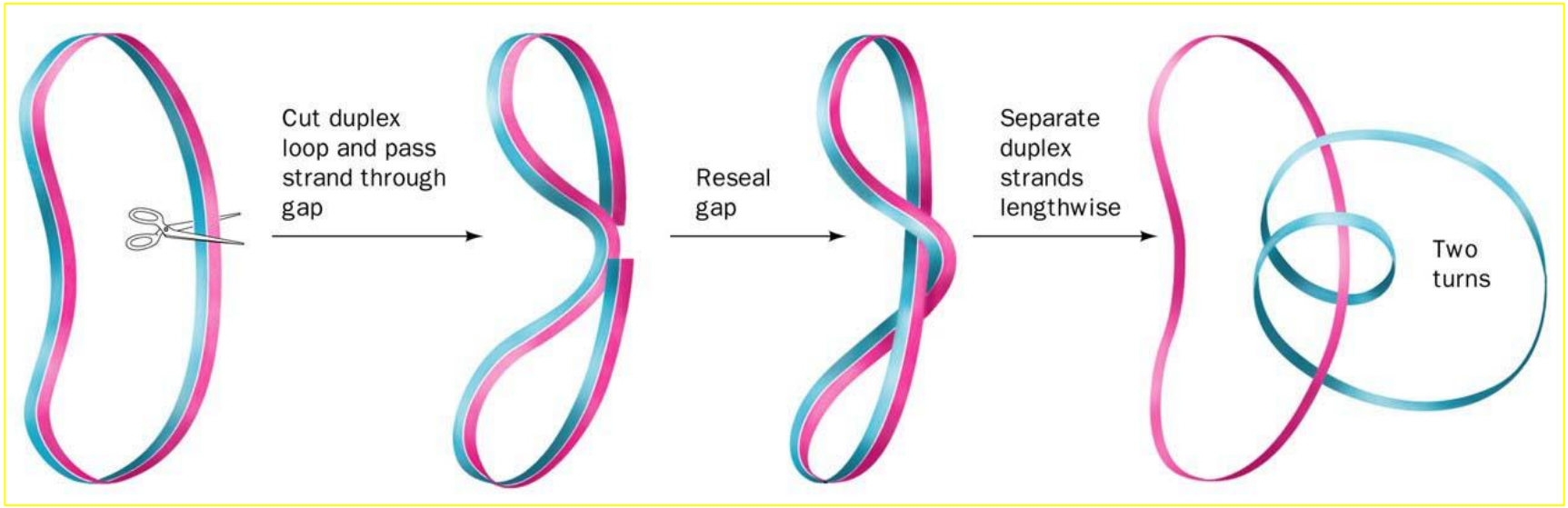
Class I DNA Topoisomerases



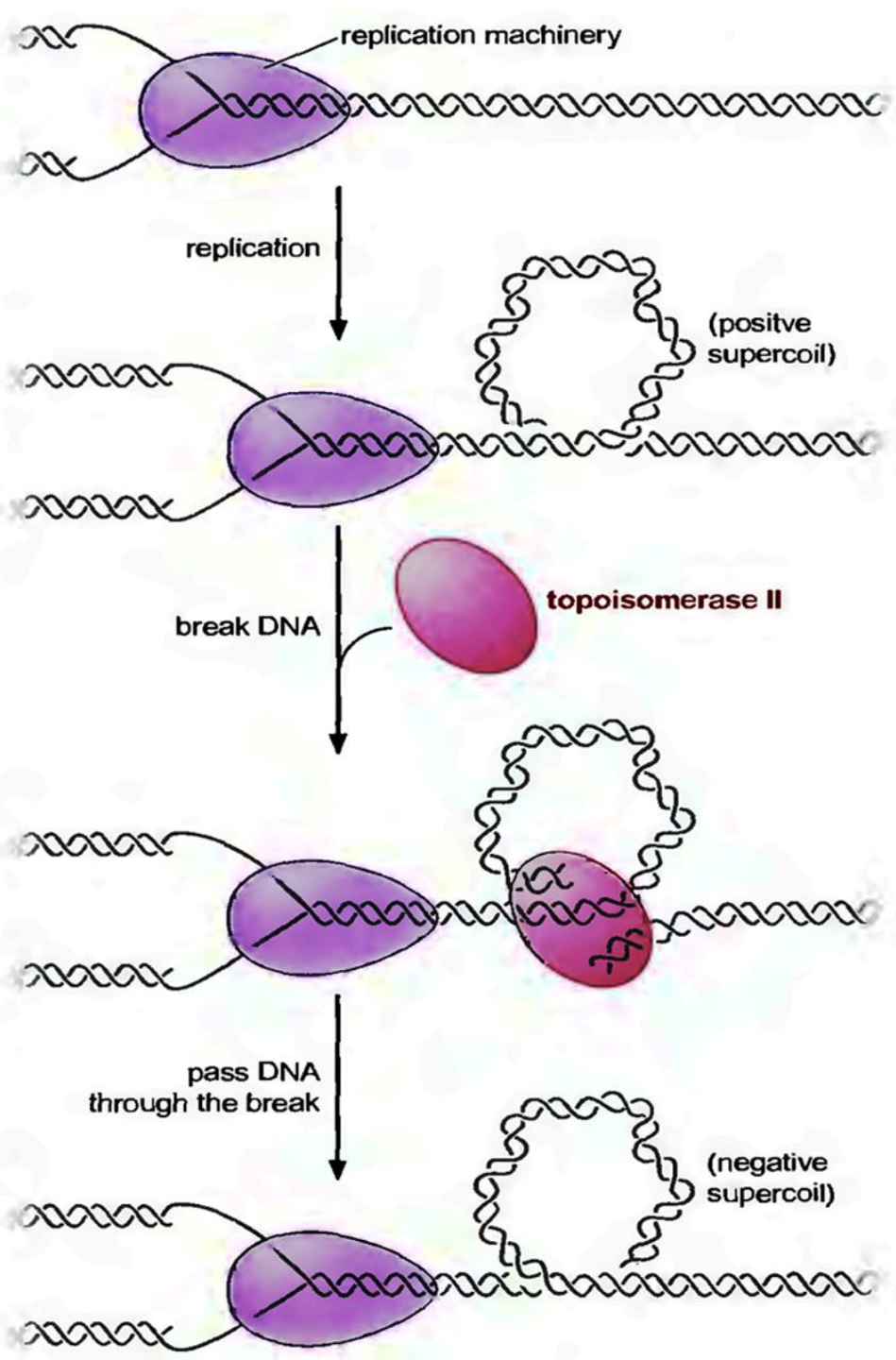
Replication-Requirements

- **Class II topoisomerases** cleave both the strands and can catenate or de-catenate covalently closed circles. **They require ATP.**
- **Mechanism:** These enzymes bind non-covalently to DNA. Both the strands are cleaved and 5'-PO_4 groups are covalently bound to tyrosine residues present in the active site. The duplex is passed through the gap facilitated by a conformational change involving ATP binding. The broken duplexes are ligated and DNA is released. Ex. Gyrase, Topoisomerase II

Class II Topoisomerase

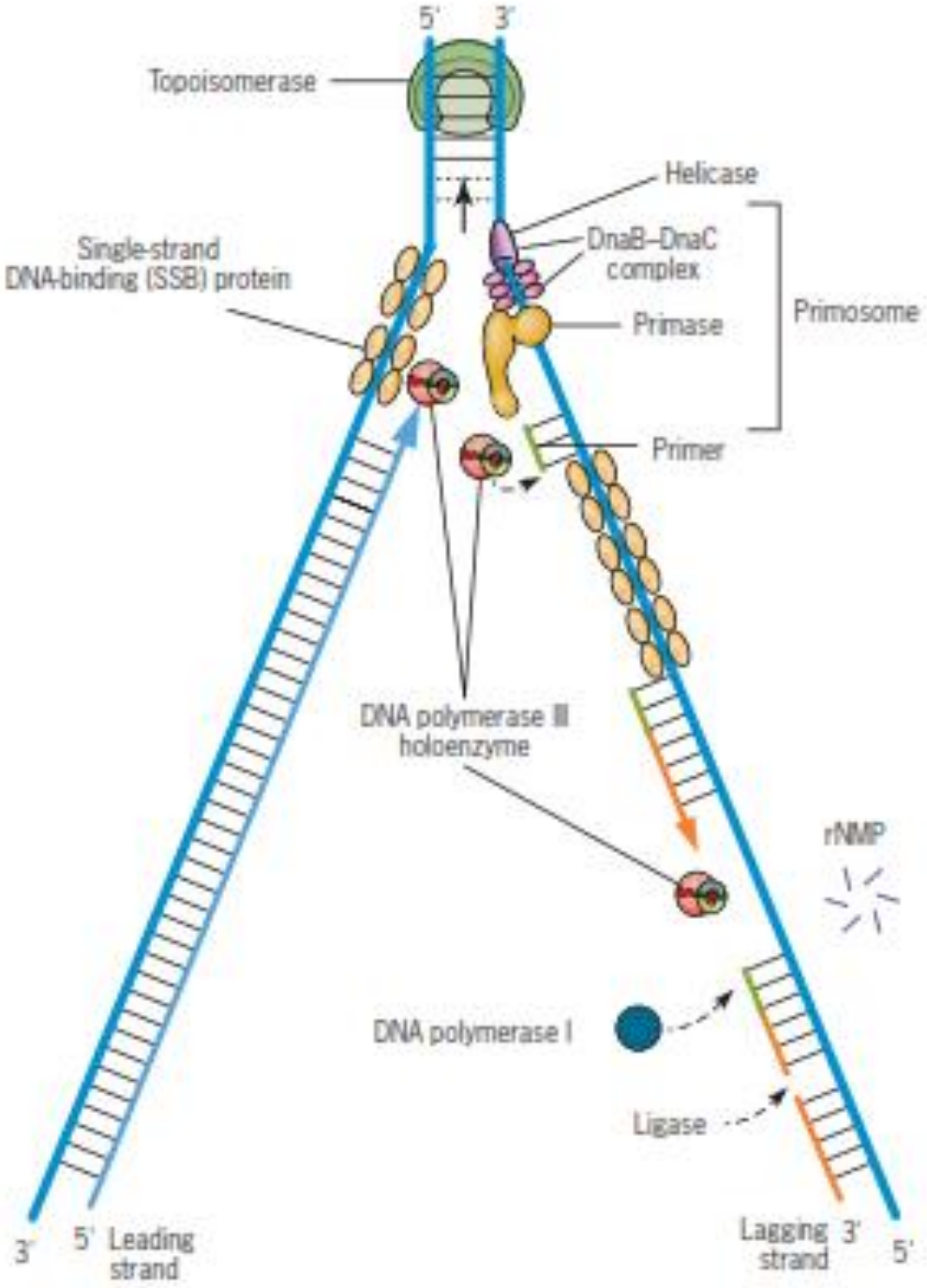


Class II topoisomerases Mechanism



Replication-Requirements

- **Single Strand Binding Protein:** Single strand binding proteins are accessory proteins which lack enzymatic activity and participate in replication process.
- **They increase the efficiency of primase in a replisome.**
- The molecular weight of a SSB protein monomer is 19KD and it is generally seen as a tetramer (so 76 KD).


5'
3'
Topoisomerase
Helicase
DnaB–DnaC complex
Primase
Primosome
Single-strand DNA-binding (SSB) protein
Primer
DNA polymerase III holoenzyme
rNMP
DNA polymerase I
Ligase
3'
5'
Leading strand
Lagging strand
3'
5'

Replication-Requirements

- The primary function of these SSB proteins is to stabilize the single stranded DNA and to prevent them from renaturation.
- They also sustain the activity of helicases, remove the secondary structures from DNA template (frame shift fidelity or frame shift accuracy is maintained) and also inhibit the nuclease action.
- **They have a high affinity for single stranded DNA but do not bind to RNA or ds DNA.**
- **They do not show any sequence specificity to binding.**

Replication-Requirements

- **Ori: “**Ori” stands for origin of replication.
- This is a unique sequence of nucleotides (DNA) where the replication is initiated.
- The origin of replication is specific to each species.
- It is a must for DNA to replicate in a host.
- If this is absent then the DNA cannot replicate.
- The origin of replication corresponding to *E. coli* is known as “Ori C”.

Replication-Requirements

- **Ori:** "OriC" is a 245bp sequence with a 9bp sequence repeated 4 times and a 13bp sequence repeated 3 times.
- This 13bp sequence is rich in AT base pairs.
- This oriC region is also rich in GATC sequence, which is seen 11 times (Generally GATC is present once in every 256bp).
- GATC sequence of oriC is a target for methylation at the N6 position of adenine by the Dam methylase.
- Methylation of GATC in both strands is essential for initiation of DNA replication as initiation is inhibited by hemimethylation of sequences of oriC.

Replication-Requirements

- **Primase:** This is an enzyme (RNA polymerase) which is a product of dnaG gene.
- This primase synthesizes the primer for lagging strand and leading strands.
- Primase is resistant to the antibiotic ***Rifampicin*** while the bacterial RNA polymerase is sensitive.
- *However in case of M13 phage the bacterial RNA polymerase is used for Primer synthesis as well.*

- There are two types of priming reaction in *E. coli*.
  (1) DnaA dependent priming initiated at OriC
  (2) PriA-dependent priming initiated at n'-*pas*
  [primosome assembly site].

- **Primosome** is a group of proteins that bind to origin site and synthesize primers to initiate replication.
- The constituents of Primosomes differ from one system to the other.
- All chromosomal DNAs have their own specific origins and their own specific primosomes. Ex: Such Primosome assemblies are found in Origin-C of *E.coli*, Col E-1 Ori, phiX174 phage origin (ori), M13 phage DNA etc.
- Primosome complex without primase referred as preprimosome complex. Based on the sequence, structures and the components assembled to form Primosomes; they are grouped in to Dna-A dependent and Pri-A dependent primosomes.

- **Dna-A dependent Primosome**: Dna-A binding leads to Dna-B and primase G binding-all together form the primosome complex. Ex: OriC of *E.coli.*
- **Pri-A dependent primosome**: This was first observed ΦX174 (ss DNA). Later it was also discovered in *E. coli & Bacillus*. The primosome complex is made up of Pri-A, Pri-B, Pri-C, Dna-T, Dna-B, Dna-C and Dna-G. The said components assemble at a particular site called "**pas**"; means **p**rimosome **a**ssembling **s**ite.
- **Note**: **PriA** dependent priming is useful to restart replication at stalled replication forks and **damaged DNA** in ***E. coli***.

- **Ter (Terminus) Sequence:**
- **Ter** is a 20bp long sequence in *E. coli* as multiple copies (7 copies TerG, TerF, TerB, TerC, TerA, TerD and TerB).
- These Ter sequences function as binding sites for a protein called Tus protein (TUS → Terminus Utilizing Substance).
- The Ter-Tus complex which has anti-helicase property can arrest the replication fork in only one direction.

- **Ter (Terminus) Sequence:**
- The first Ter-Tus complex encountered by any replication fork will be functional.
- The second replication fork halts when both of them meet.
- The final few hundred base pairs of DNA between these two large protein complexes are then replicated by yet another mechanism which is not clearly understood.

- **Replication of *E. coli* chromosome**
- The synthesis of a DNA molecule can be decided into 3 stages viz., initiation, elongation and termination.
- Each stage is different from the others in both, the enzymes involved and the biochemical reactions that are taking place.

- **Initiation:**
- At least 9 different enzymes or proteins participate in the initiation phase of replication.
- They open the DNA and establish a pre priming complex for subsequent reaction.
- They include Dna A protein (initiator protein), Dna B Protein (helicases), Dna C protein (helicases loader), Dna G protein(primase), HU (Histone like) protein, SSB Protein, Gyrase (topoisomerase), Dam methylase and RNA polymerase which facilitates Dna A activity.

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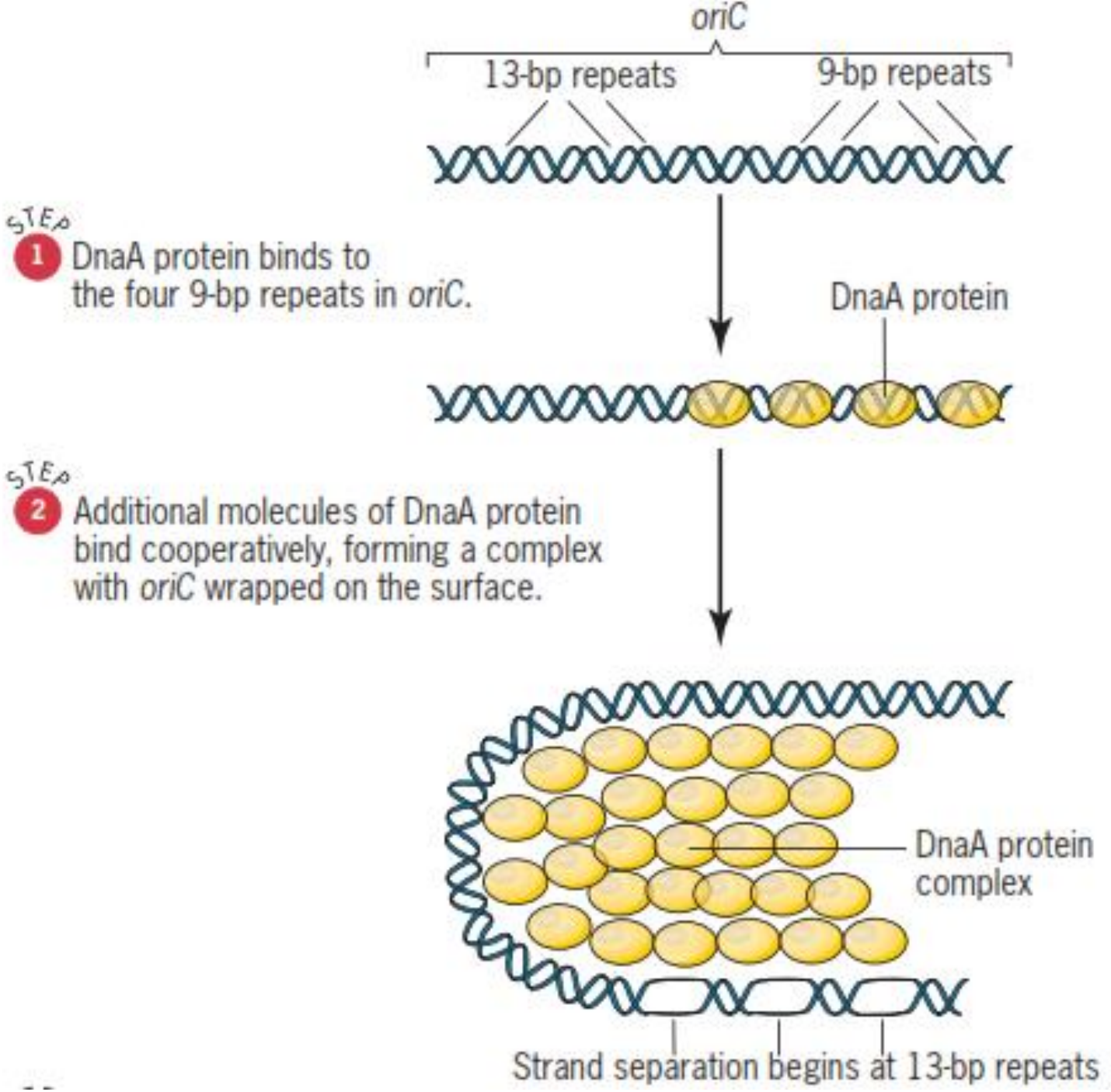
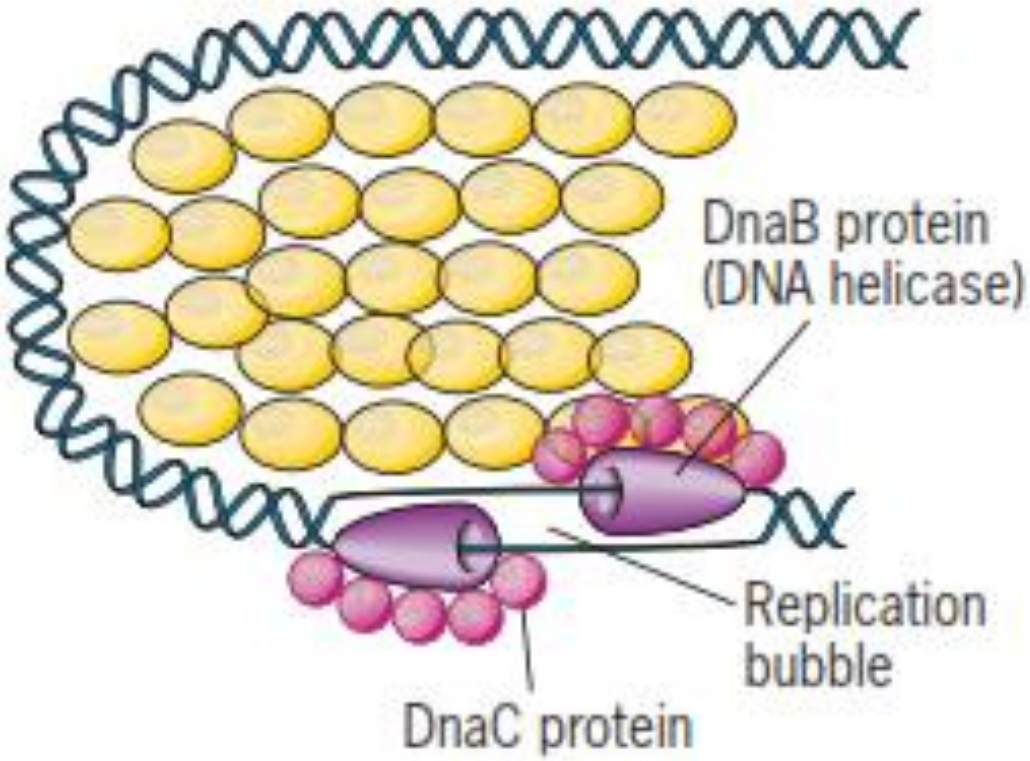
- **Initiation:**
- The crucial component in the initiation process is the Dna A protein.
- A single complex of 4 – 5 Dna A proteins will initially bind to four 9 bp repeat sequences of OriC.
- The number soon increases to 20 – 40, which also bind and successively denature the three 13bp repeats of OriC, rich in A=T bp.
- This step requires ATP and HU protein.
- Dna A protein also has a role in the regulation of initiation.
- Dna A protein melts DNA in an ATP dependant process. (Dna A protein bound to ATP is active and free Dna A protein is inactive)
- Synthesis of DnaA protein is coupled to growth rate so that the initiation of replication is also coupled to growth rate.

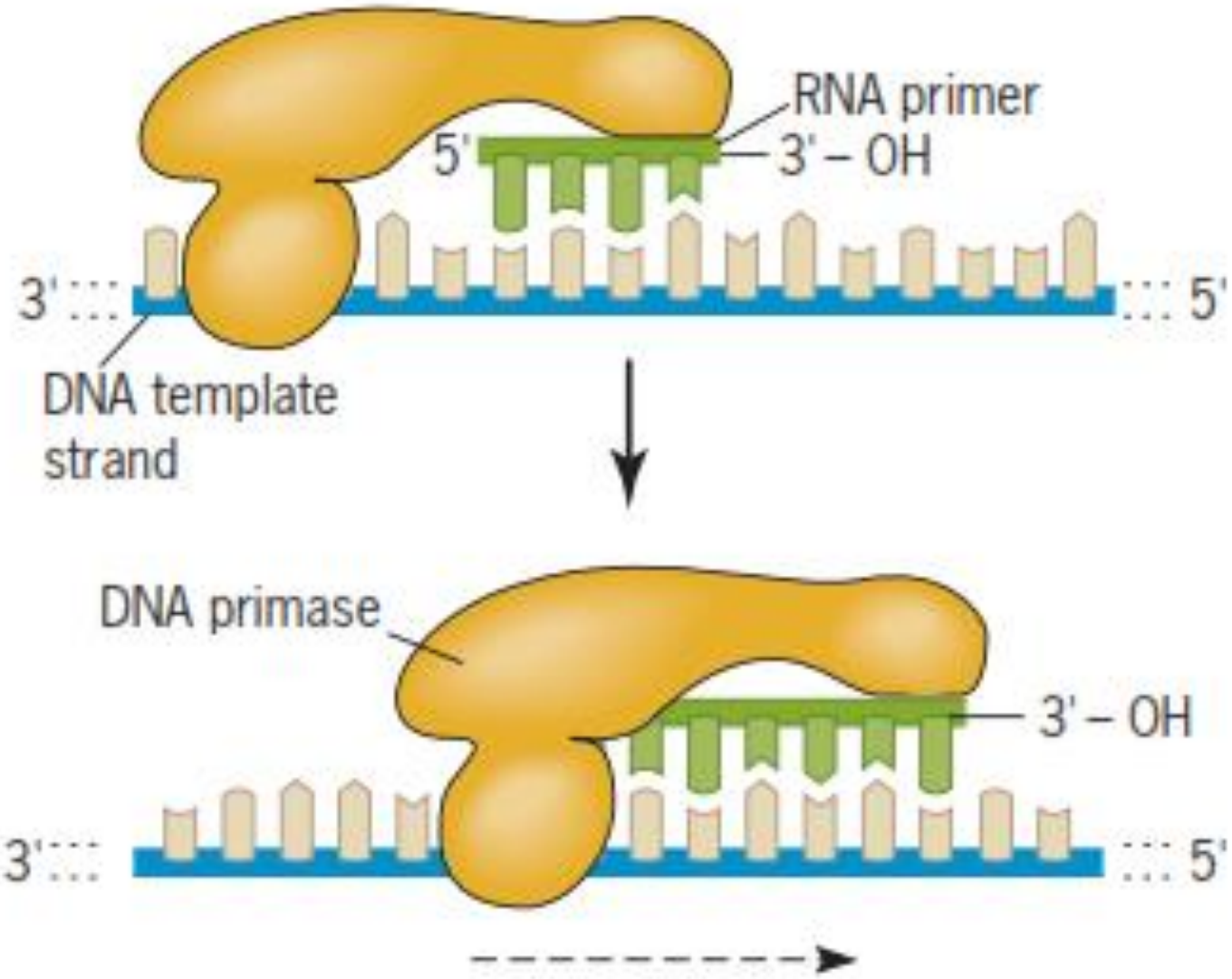

oriC
13-bp repeats
9-bp repeats
STEP 1
DnaA protein binds to the four 9-bp repeats in oriC.
DnaA protein
STEP 2
Additional molecules of DnaA protein bind cooperatively, forming a complex with oriC wrapped on the surface.
DnaA protein complex
Strand separation begins at 13-bp repeats

STEP 3 DnaB protein (DNA helicase) and DnaC protein join the initiation complex and produce a replication bubble.



- **Initiation:**
- The DnaC protein (helicase loader) along with DnaB protein (helicase) binds to this unwound single stranded region at the origin.
- The helicase loader then directs the assembly of its associated DNA helicase around the ssDNA.
- Upon completion of this task, the helicase loader is released activating the helicase.
- Thus two hexamers of DnaB are loaded, one on to each strand to act as helicases, unwind the DNA in both directions (bidirectional) and create 2 replication forks.
- The tension developed in the DNA is released by topoisomerases (DNA gyrase).
- The single strand binding proteins binds to the ssDNA stabilizing them and also prevents them for renaturation.
- There are several interactions between Proteins at Replication Fork form the *E. coli* Replisome.

- **Initiation:**
- Each DNA **helicase** recruits a DNA primase which synthesizes an RNA primer on each template.
- The movement of the DNA helicases also removes any remaining DnaA bound to the replicator.
- The newly synthesized primers are recognized by the clamp loader components of two DNA Pol III holoenzymes.
- Sliding clamps are assembled on each RNA primer, and leading strand synthesis is initiated by one of the two core DNA Pol III enzymes of each holoenzyme.


RNA primer
5'
3' – OH
3'
5'
DNA template strand
DNA primase
3' – OH
3'
5'

- **Initiation:**
- After each DNA helicase has moved approximately 1,000 bases, a second RNA primer is synthesized on each lagging strand template and a sliding clamp is loaded.
- The resulting primer template junction is recognized by the second DNA Pol III core enzyme in each holoenzyme, resulting in the initiation of lagging strand synthesis.
- Leading and lagging strand synthesis is now initiated at each replication fork and continues to the end of the template or until another replication fork from an adjacent origin of replication is reached.
- Initiation is the only stage that can be regulated in replication.

Elongation:

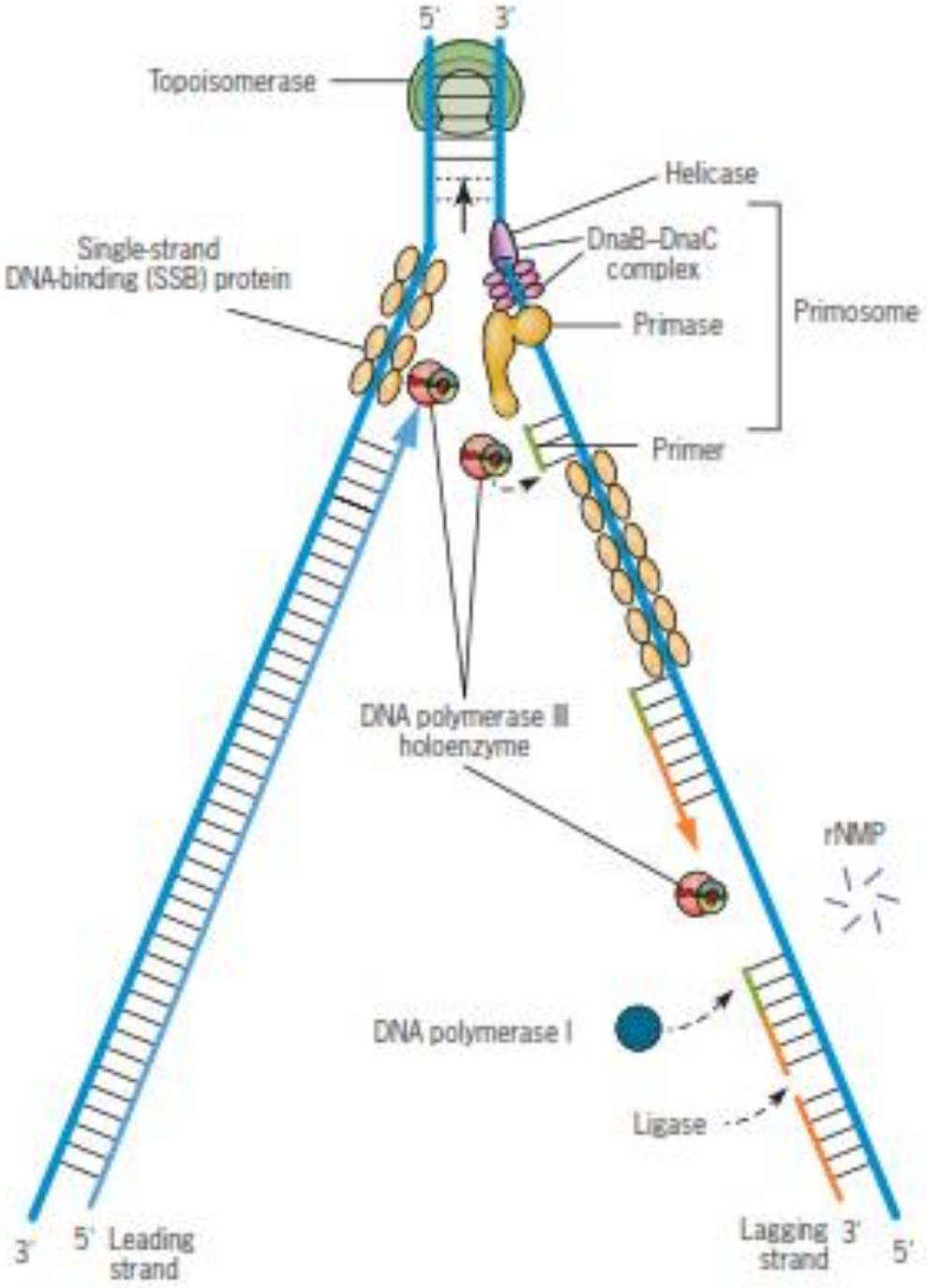
- Leading strand synthesis and lagging strand synthesis take place in this.
- There are some similarities and differences between the syntheses of both strands.
- DNA helicases unwind the DNA, topological strain is released by topoisomerases, and each separate strand is stabilized by SSB proteins.
- These processes are common to both the leading and lagging strands.

Elongation:

- DNA is anti-parallel in nature and DNA replicates in a semi conservative mode.
- DNA polymerases observed till today polymerize only in 5→3 direction.
- During replication DNA will undergo denaturation thereby producing 2 templates.
- One template has 3’ end and the other has 5’ end.
- A primer will be synthesized opposite to the 3’ end of the template and will be extended by DNA polymerase.
- The synthesis of this strand takes place continuously.
- This strand is known as leading strand as the number of nucleotides that have been polymerized in this strand are always more than the other.

Elongation:

- **Leading strand synthesis:**
- Primase synthesizes the primer using the 3’ terminus of the template.
- After an RNA primer is synthesized, the primase is released from the DNA helicase.
- This primer (10 to 60 nucleotides) is extended by DNA pol III using nucleoside triphosphates thus leading strand synthesis takes place continuously in 5`→3` direction.


5'
3'
Topoisomerase
Helicase
DnaB–DnaC complex
Primase
Primosome
Single-strand DNA-binding (SSB) protein
Primer
DNA polymerase III holoenzyme
rNMP
DNA polymerase I
Ligase
3'
5'
Leading strand
Lagging strand
3'
5'

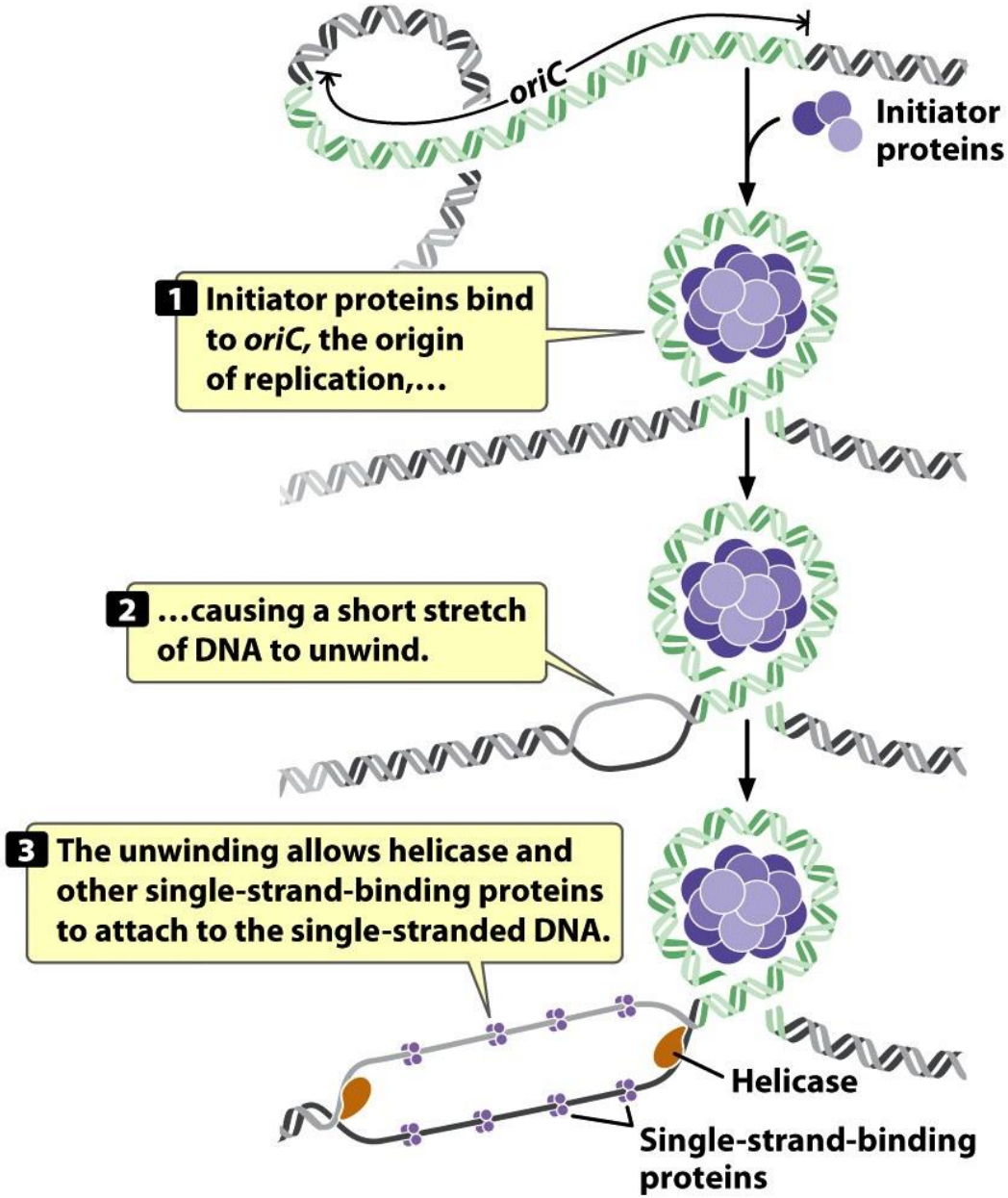


Figure 12-11
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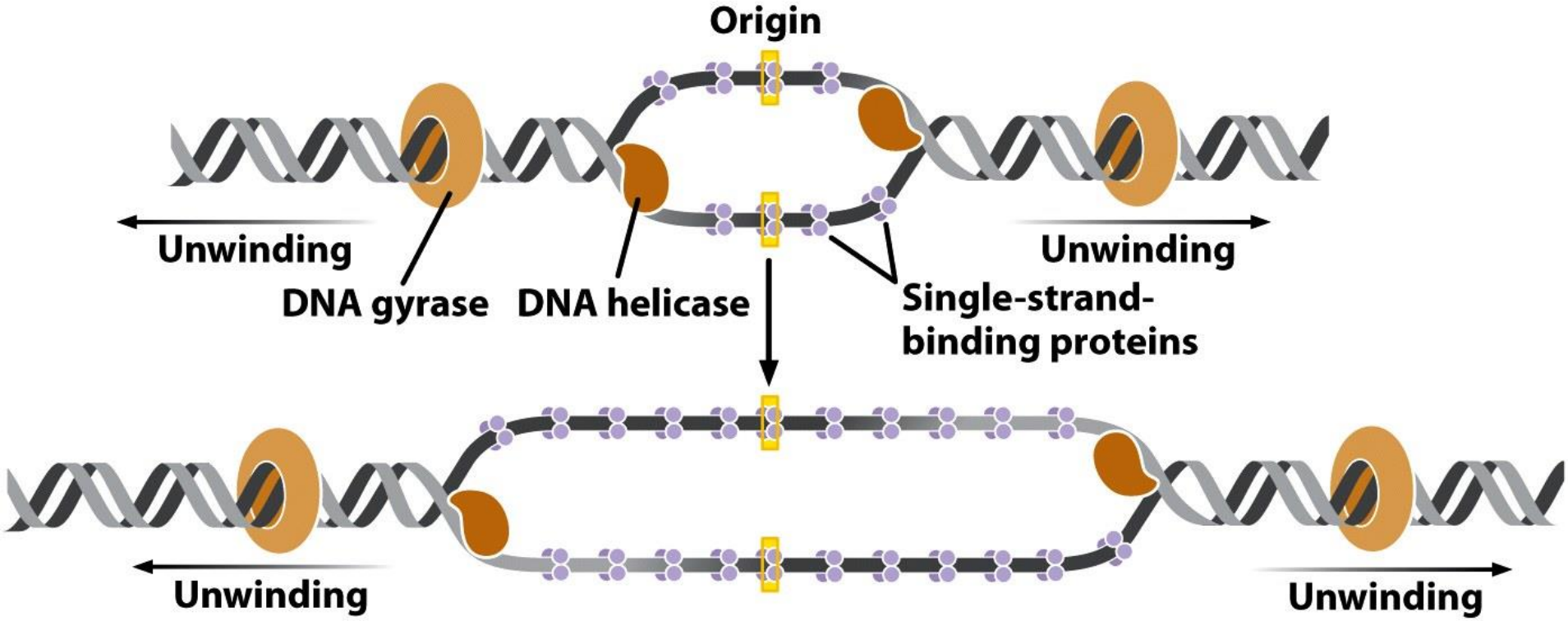


Figure 12-12
Genetics: A Conceptual Approach, Third Edition

Elongation:

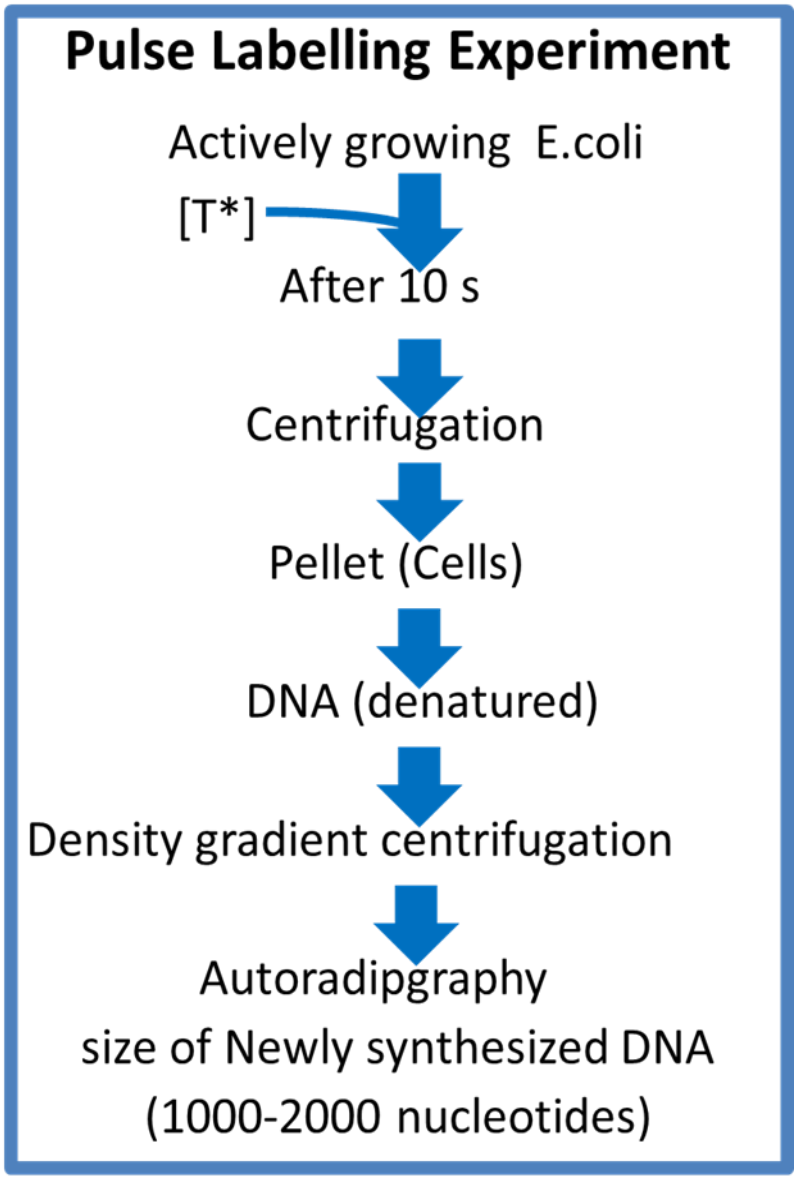
- **Lagging strand synthesis:**
- How DNA is replicated in the second strand (lagging strand) was not well understood initially.
- Reiji Okazaki and his coworkers proposed **Discontinuous model of replication** to explain DNA synthesis in the second strand (lagging strand).

Discontinuous model of replication

- According to this model DNA in leading strand will be continuously synthesized.
- But in the lagging strand DNA is synthesized in the form of fragments.
- These fragments will later unite to form a continuous strand.
- To prove discontinuous (also known as semi discontinuous) model of replication, Okazaki and his coworkers have carried out Pulse Labeling and Pulse Chase Experiments or Okazaki Experiments.

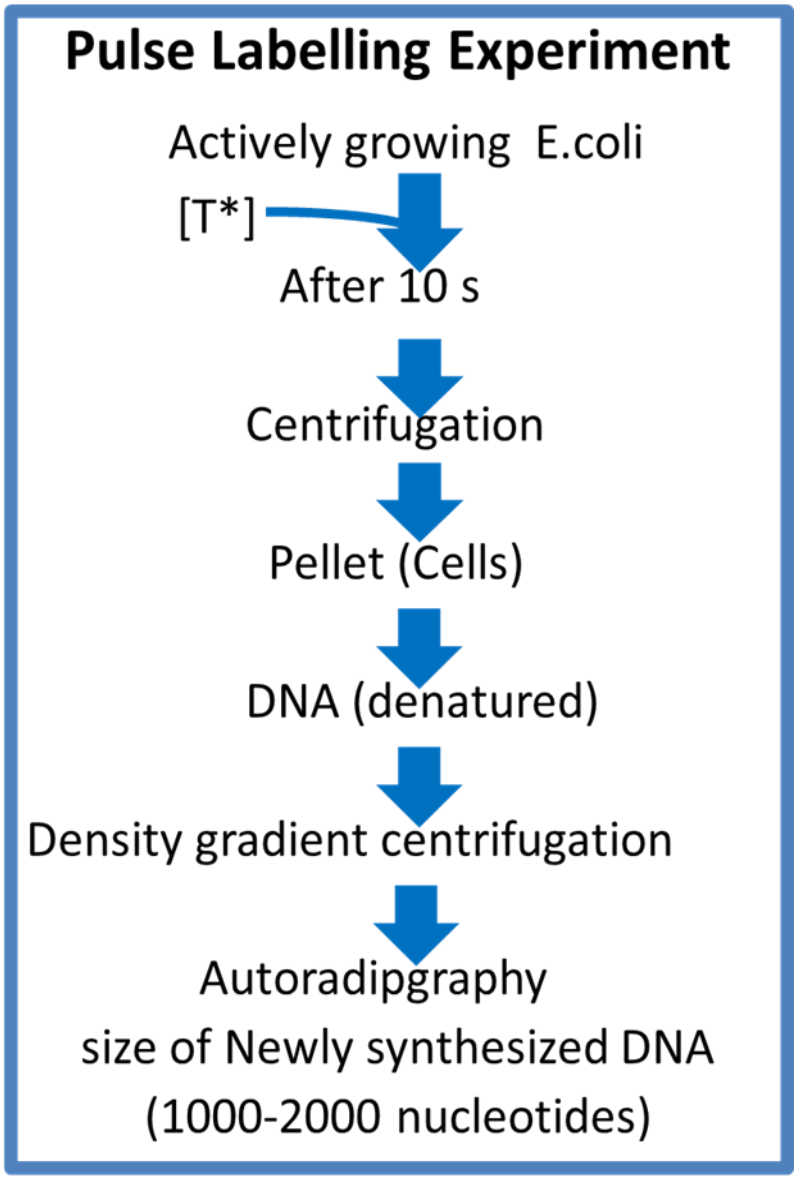
Pulse Labeling Experiments

- To an actively growing culture of *E. coli* radio labeled Thymine (tritium) was added. So that all newly synthesized DNA is radiolabeled.
- After 10 seconds *E. coli* cells separated from media, DNA was isolated, denatured and subjected to density gradient centrifugation to separate DNA based on their size.



Pulse Labeling Experiments

- The separated DNA fragments were analyzed for radioactivity (only newly synthesized DNA exhibits).
- Size of radioactive DNA fragments is in between 1000 – 2000 nucleotides.
- These fragments are known as "**Okazaki fragments**".
- From these results it may be concluded that newly synthesized DNA, which is radio labeled is synthesized in the form of fragments.



Pulse Chase Experiments

Actively growing E. coli cells

After 10 seconds

centrifugation

Pellet

Constituted in normal

Medium

Cells

Allowed to grow in normal medium

Some Cells were taken at regular intervals (1', 2' 3' …)

From isolated cells DNA was isolated, denatured and subjected to density gradient Centrifugation

Autoradiography

With the increases of time the length of radiolabeled DNA is increasing

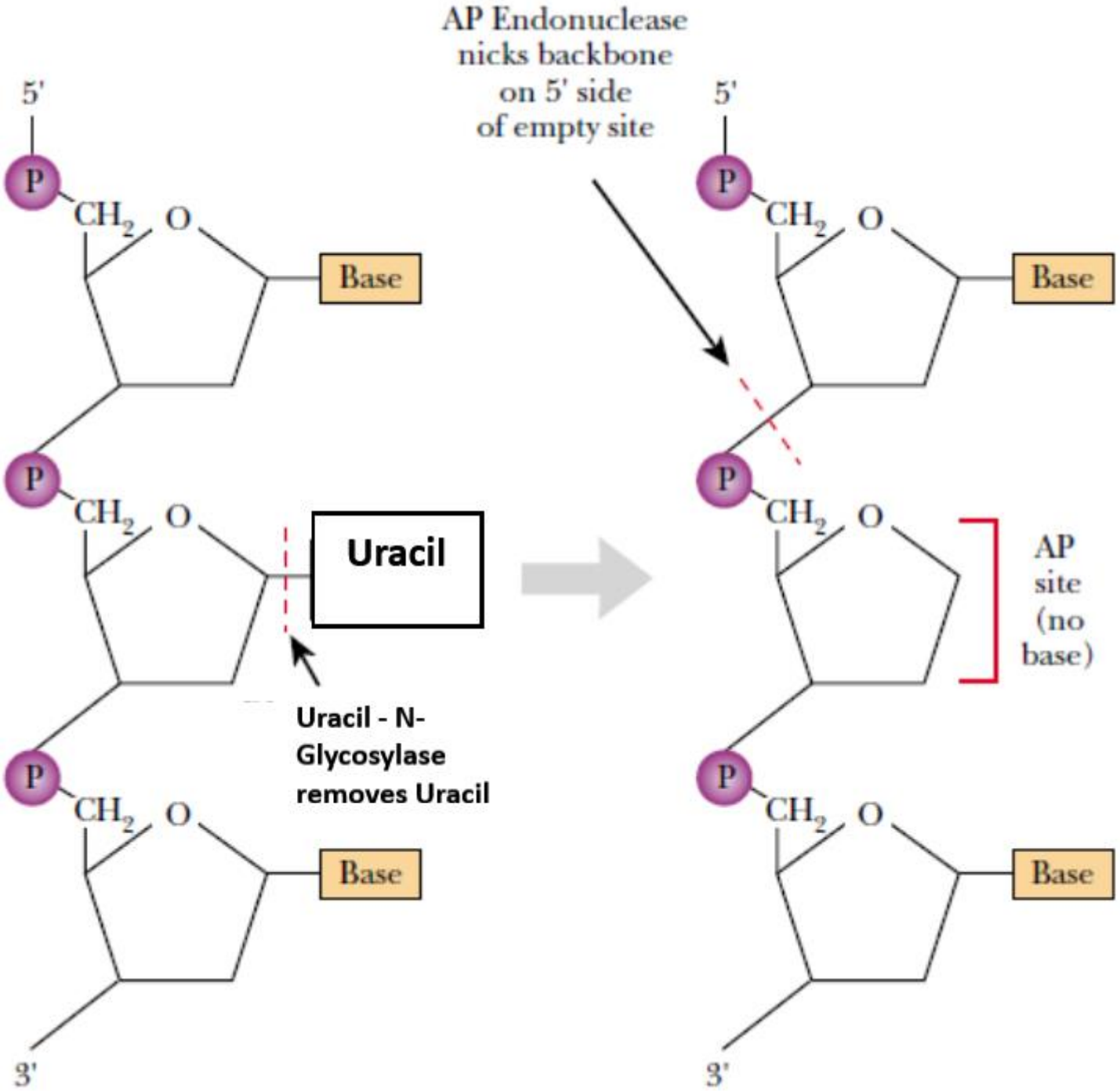
Pulse Chase Experiment

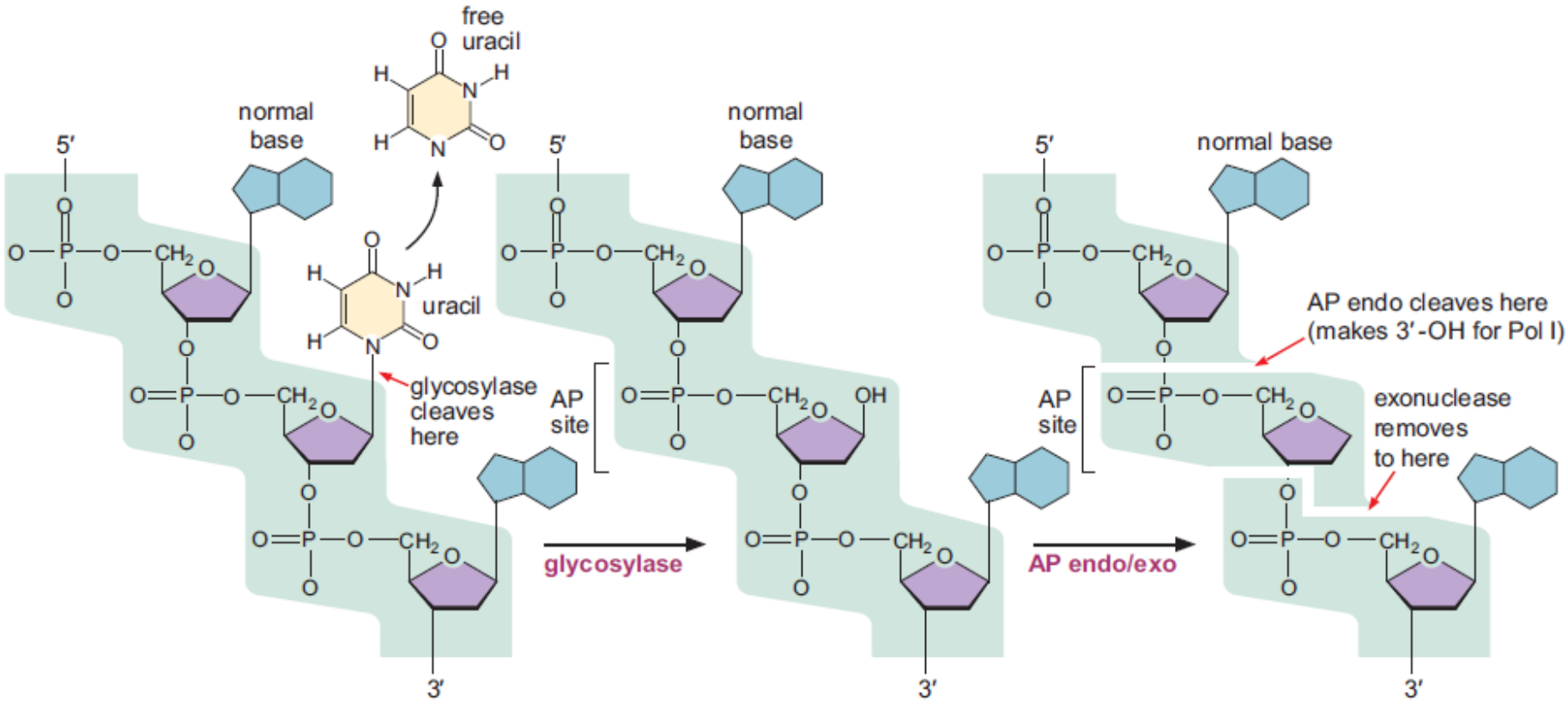
- **Pulse Chase Experiment** is an extension of pulse labeling experiment to know the status of the radio labeled DNA.
- In this again a culture of *E. coli* was actively growing in a medium.
- To this medium radio labeled Thymine (Tritium, $^{3}H_{1}$) was added. After 10 seconds the radio labeled thymine in the medium was replaced with normal thymine.
- So that DNA synthesized only during the 10 seconds is radio labeled.
- The cells were maintained in the normal medium and from time to time the cells were removed and their DNA was isolated, denatured and subjected to density gradient centrifugation.
- The separated DNA fragments were analyzed for radioactivity.

Pulse Chase Experiment

- **Pulse Chase Experiment:**
- As the time of maintaining the cells in normal medium increased the length of radio labeled DNA fragments has increased.
- This is possible only when DNA fragments generated during the 10 second time are **joined**.
- So in lagging strand, the DNA is initially **synthesized** in the form of short **fragments** which are then joined to form a long strand.
- So this conclusively proves the discontinuous mode of replication.
- When the same experiment was repeated with eukaryotes similar results were obtained.
- However the lengths of Okazaki fragments vary from 100 – 200 in eukaryotes.

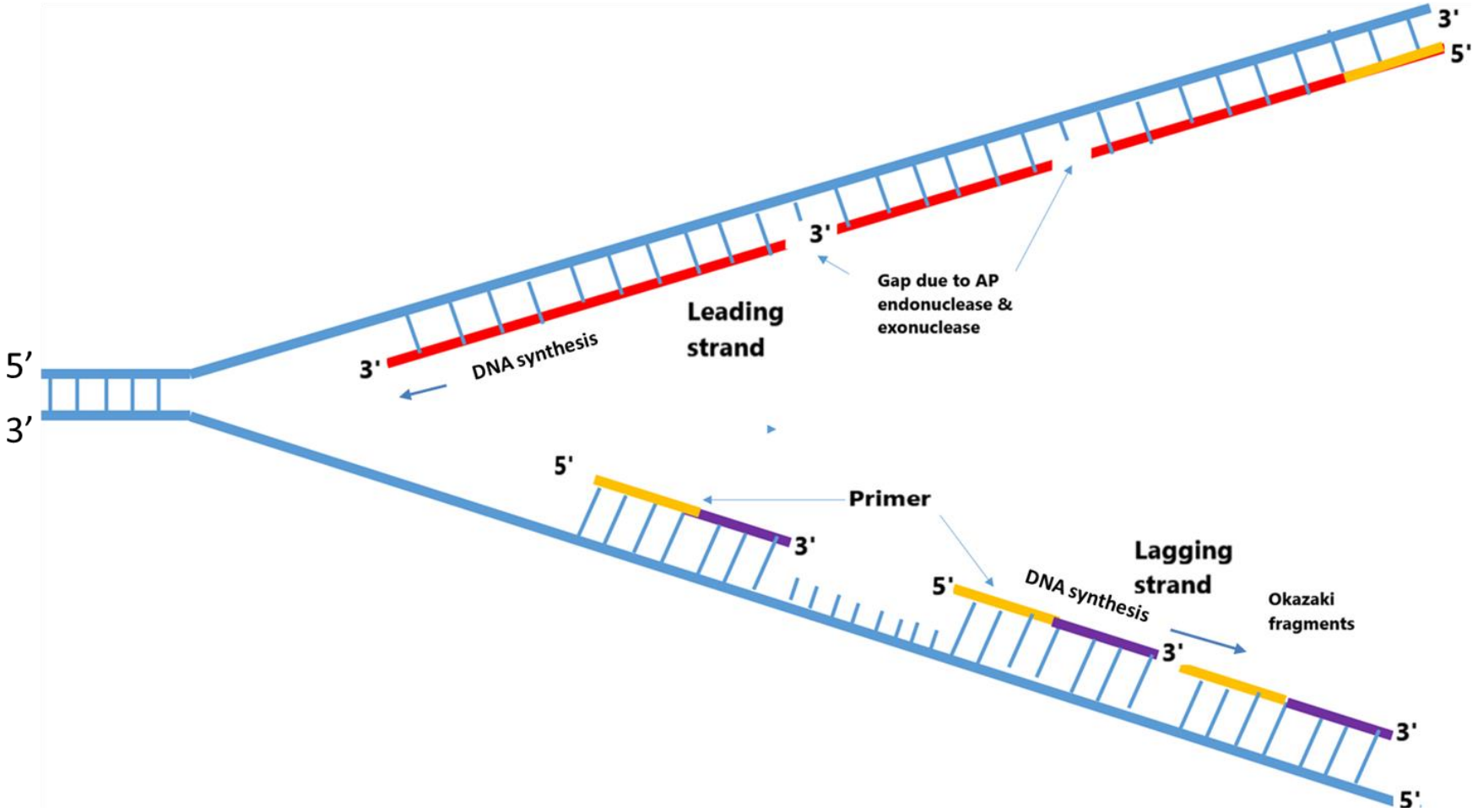
- DNA in case of lagging strand is synthesized discontinuously but leading strand is continuously synthesized.
- Then why the radio labeled DNA is seen only in fragments of 1000 – 2000 nucleotides but not in long DNA (leading strand).
- The plausible explanation:
- DNA polymerases cannot **distinguish** between **dTTP** and **dUTP**.
- Hence occasionally uracil will be incorporated in the place of **thymine** .
- However when uracil is incorporated immediately the enzyme uracil – N- glycosylase, breaks the glycosidic bond that links uracil with deoxyribose thereby producing apyrimidinic site (AP site) in DNA.
- Such AP sites are cleaved by the enzyme AP endonuclease and an associated exonuclease creates a gap which will be filled up by DNA polymerase and then sealed by DNA ligase.
- Such events are common during DNA replication.
- Hence during pulse labeling experiments though DNA is continuously synthesized in leading strand it appears to be synthesized only in the form of fragments.


AP Endonuclease
nicks backbone
on 5' side
of empty site
5'
P
CH2
O
Base
Uracil
Uracil - N-
Glycosylase
removes Uracil
AP
site
(no
base)
3'



- **Uracil glycosylase** hydrolyzes the **glycosidic** bond to release **uracil** from the **DNA** backbone to leave an **AP site** (apurinic or, in this case, apyrimidinic site). **AP endonuclease** cuts the DNA backbone at the 5’ position of the AP site, leaving a 3’OH; exonuclease cuts at the 3’ position of the AP site, leaving a 5’-phosphate. The resulting gap is filled in by DNA Pol I.

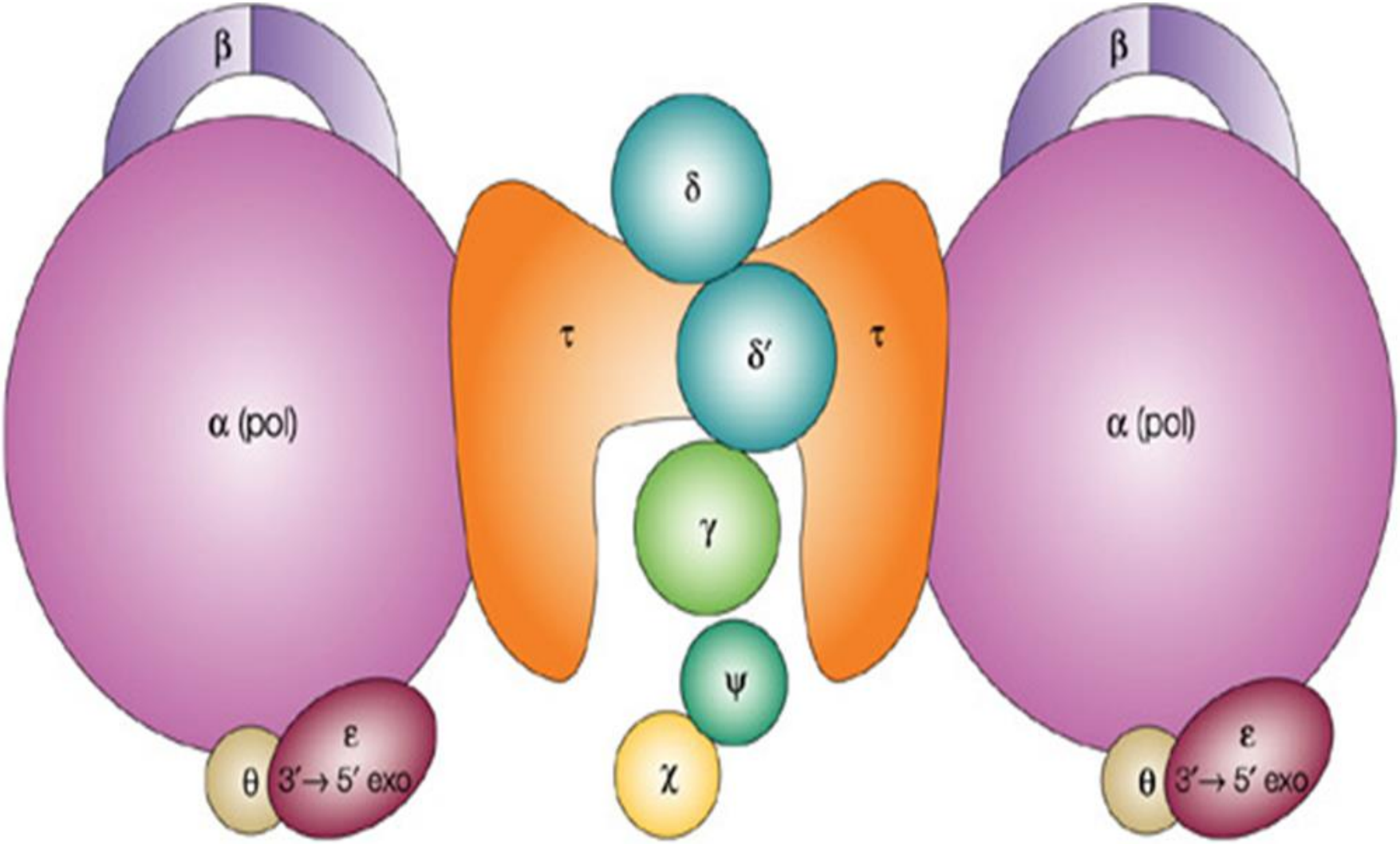
Replicating DNA



Elongation

- Both leading and lagging strands are elongated by DNA polymerase III holoenzyme which is a heterodimer.
- The synthesis of leading and lagging strands is coordinated by the heterodimer, DNA Pol III holoenzyme.
- The α unit of DNA pol III polymerizes and ε subunit exhibits the 3`→5` exonuclease activity to maintain the fidelity of replication.
- The γ complex (f γ, δ, δ`, χ and ψ) dissociates the β subunit from holoenzyme wherever the holoenzymes meets 5` end of primer. The enzyme hops to the next available primer that can be extended where it rejoins or re-associates with β subunit and continues the DNA polymerization.
- DNA pol I replaces the RNA primer with DNA strand by simultaneously exhibiting 5`→3` exonuclease activity & 5`→3` polymerization.
- Finally the nick is sealed by DNA ligase.

DNA Pol III



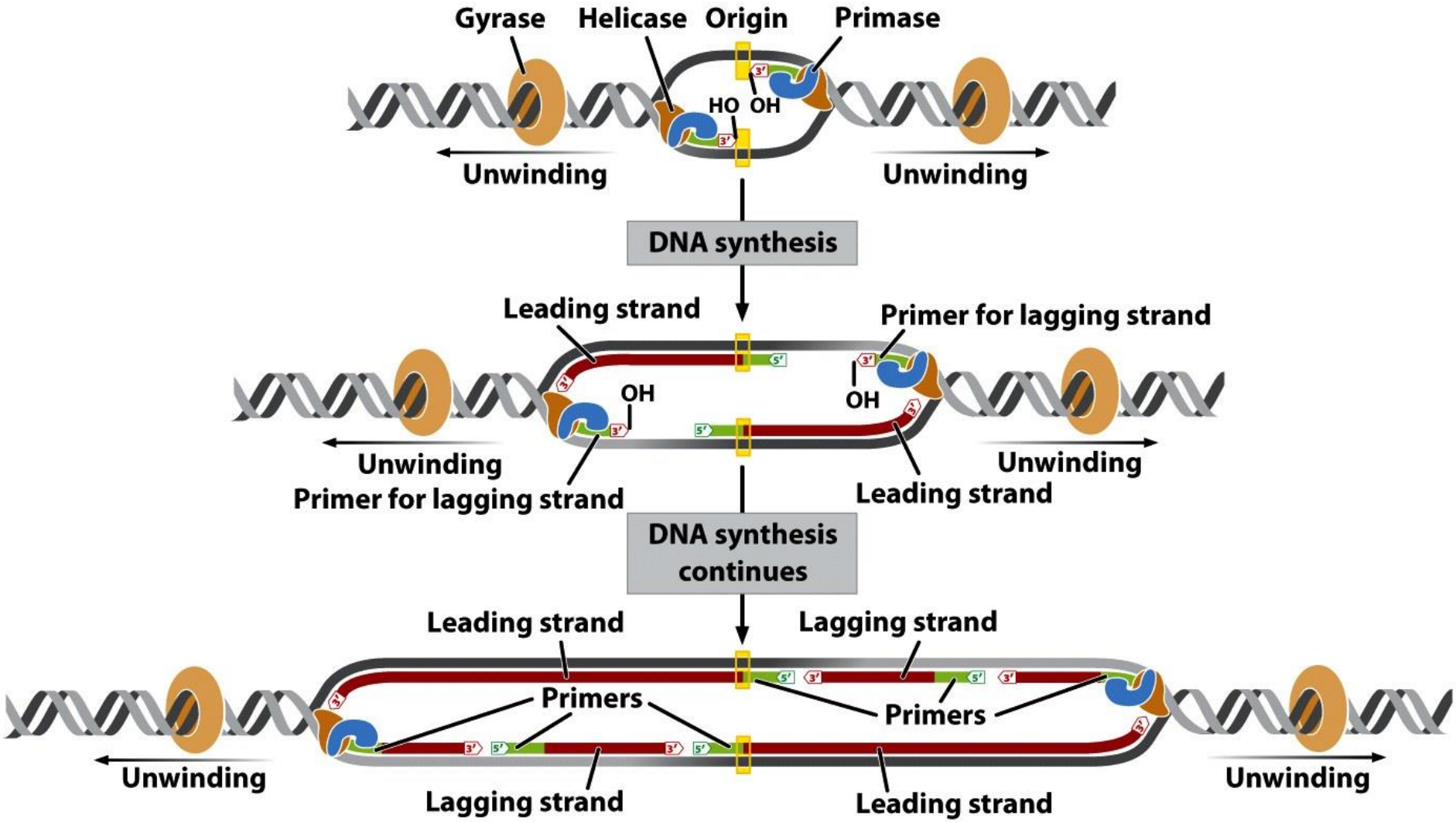


Figure 12-13
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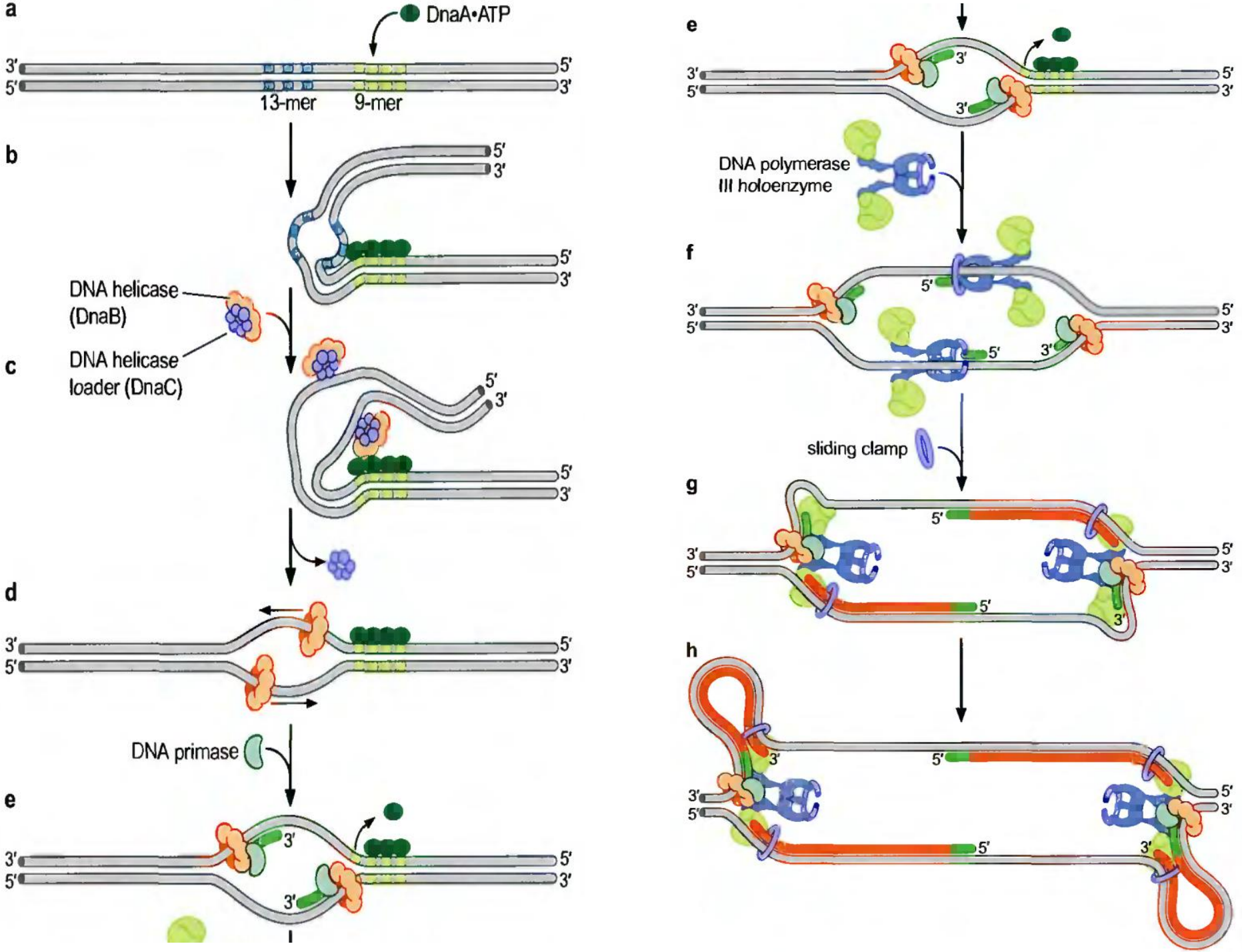
a
DnaA•ATP
3′
5′
5′
3′
13-mer
9-mer
b
5′
3′
5′
3′
DNA helicase
(DnaB)
DNA helicase
loader (DnaC)
c
5′
3′
5′
3′
d
3′
5′
5′
3′
DNA primase
e
3′
3′
3′
5′
5′
3′
e
3′
3′
3′
5′
5′
3′
DNA polymerase
III holoenzyme
f
5′
3′
5′
5′
3′
5′
3′
sliding clamp
g
5′
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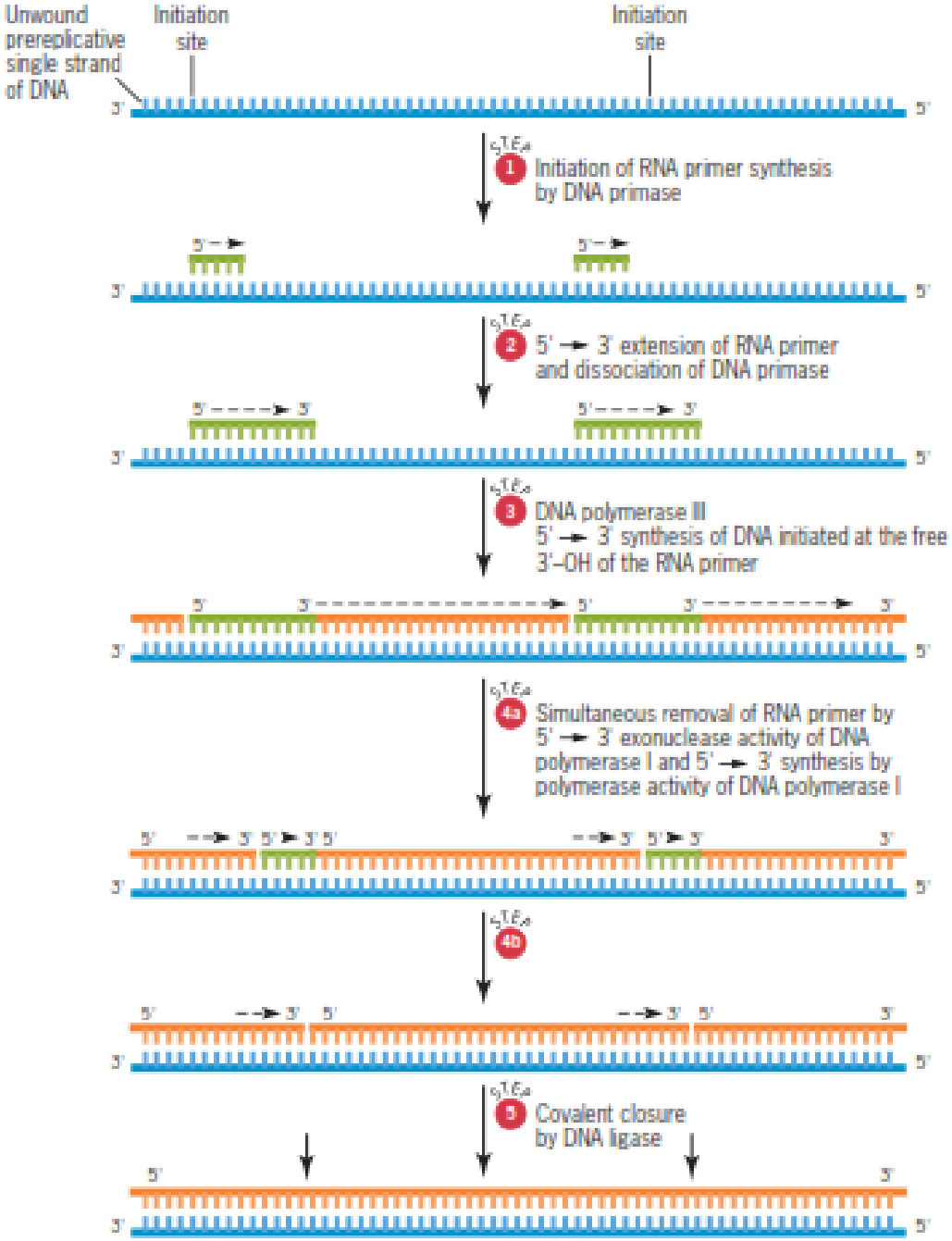
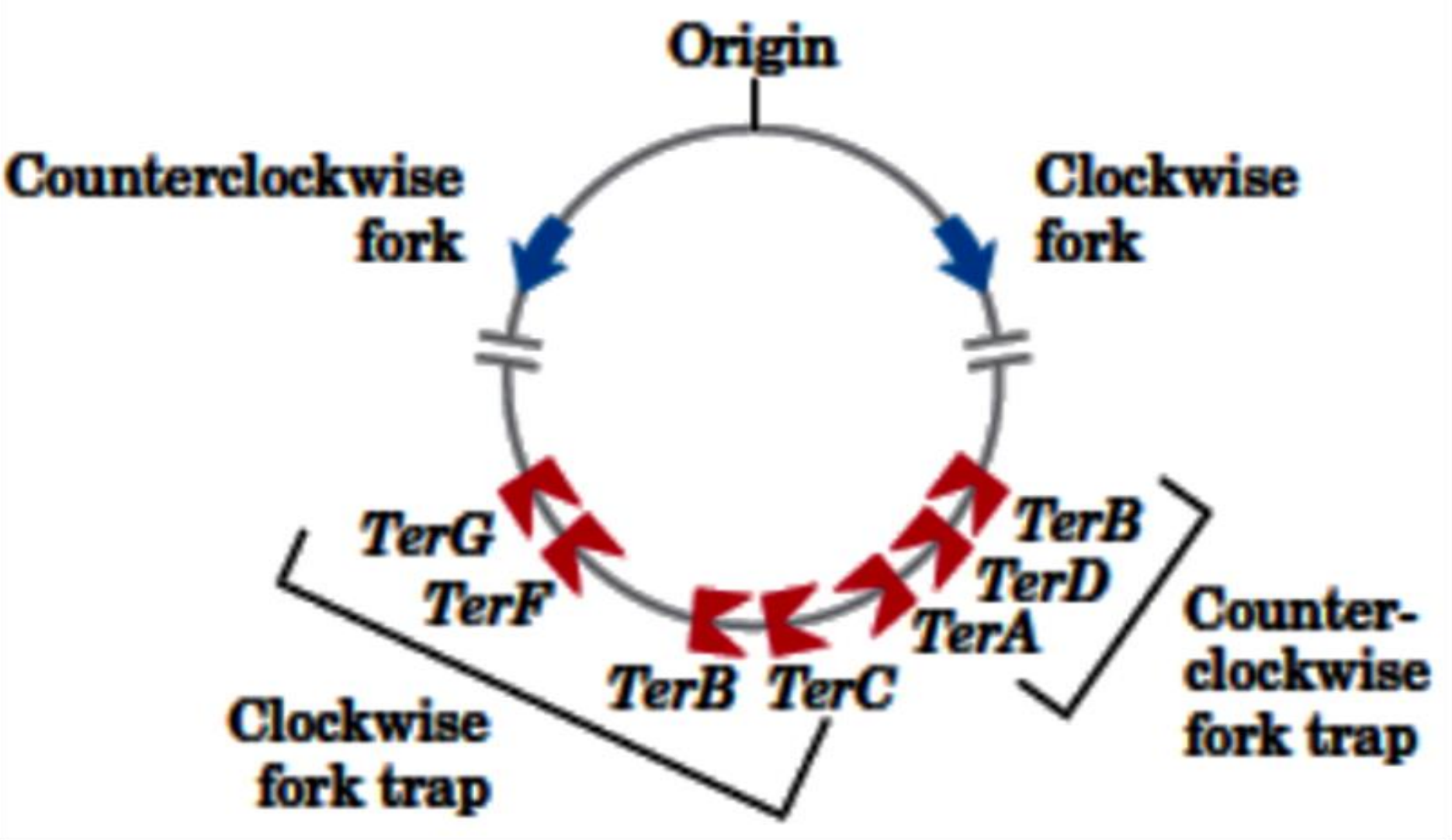
5′
5′
3′
5′
3′
5′
3′


Unwound prereplicative single strand of DNA
Initiation site
Initiation site
5'
3'
1 Initiation of RNA primer synthesis by DNA primase
2 5' → 3' extension of RNA primer and dissociation of DNA primase
3 DNA polymerase III 5' → 3' synthesis of DNA initiated at the free 3'–OH of the RNA primer
4a Simultaneous removal of RNA primer by 5' → 3' exonuclease activity of DNA polymerase I and 5' → 3' synthesis by polymerase activity of DNA polymerase I
4b
5 Covalent closure by DNA ligase

Termination

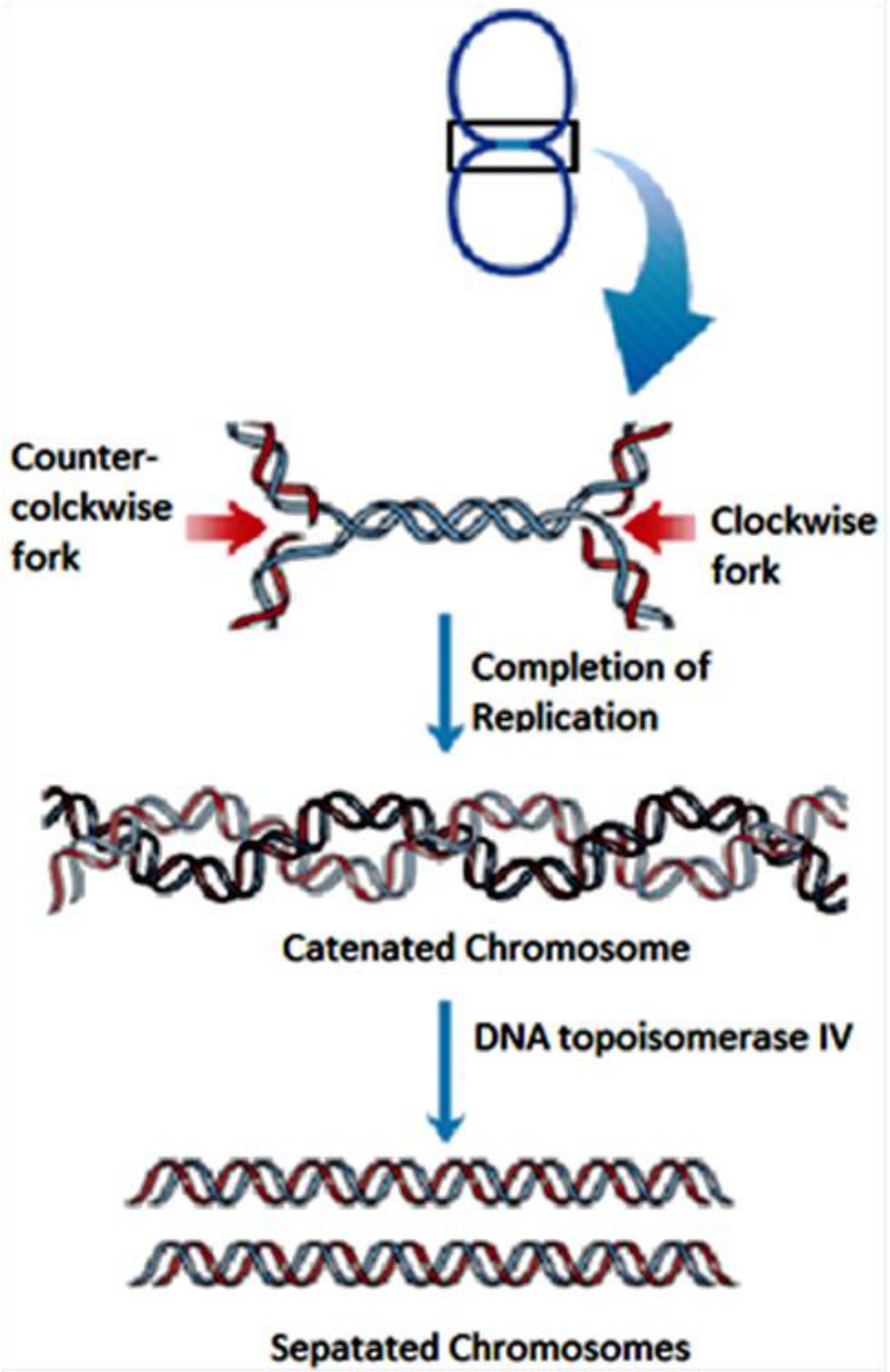
- The two replication forks meet approximately 180^{o} opposite to ori C.
- Around this region are found *ter* sites to which the *tus* protein binds and this complex exhibits anti-helicase property or inhibits the DnaB helicase.
- However it is not clearly understood how the final few hundred nucleotides are replicated to complete replication.

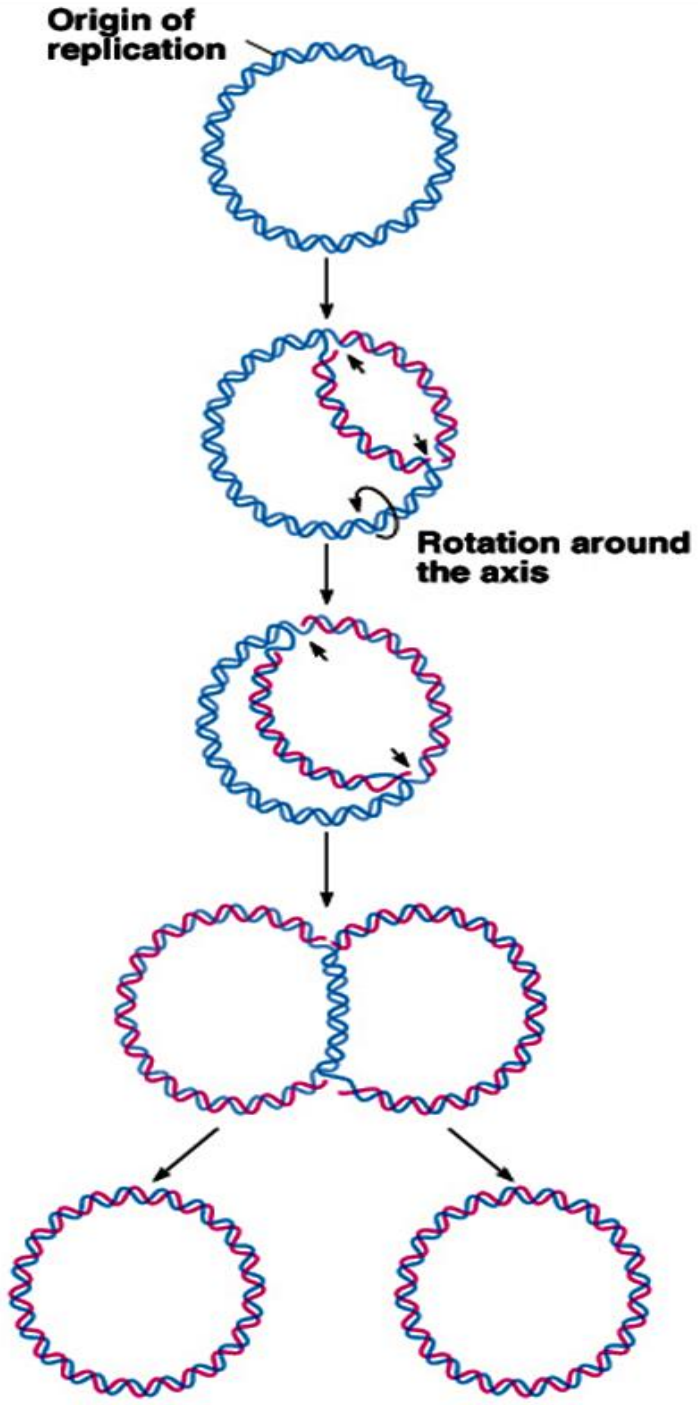


The Ter sequences are positioned on the chromosome in two clusters with opposite orientations.

Termination

- Once replication is completed the two daughter circles remain interlinked (catenated).
- They are unlinked in *E. coli*, by a type II topoisomerases, the **topoisomerase IV** by **decatenation** process.
- In decatenation **both** DNA strands of one **chromosome** are transiently **broken**, the other **chromosome** is **passed** through the **break** and then the break is **sealed** thus separating the replicated chromosomes.

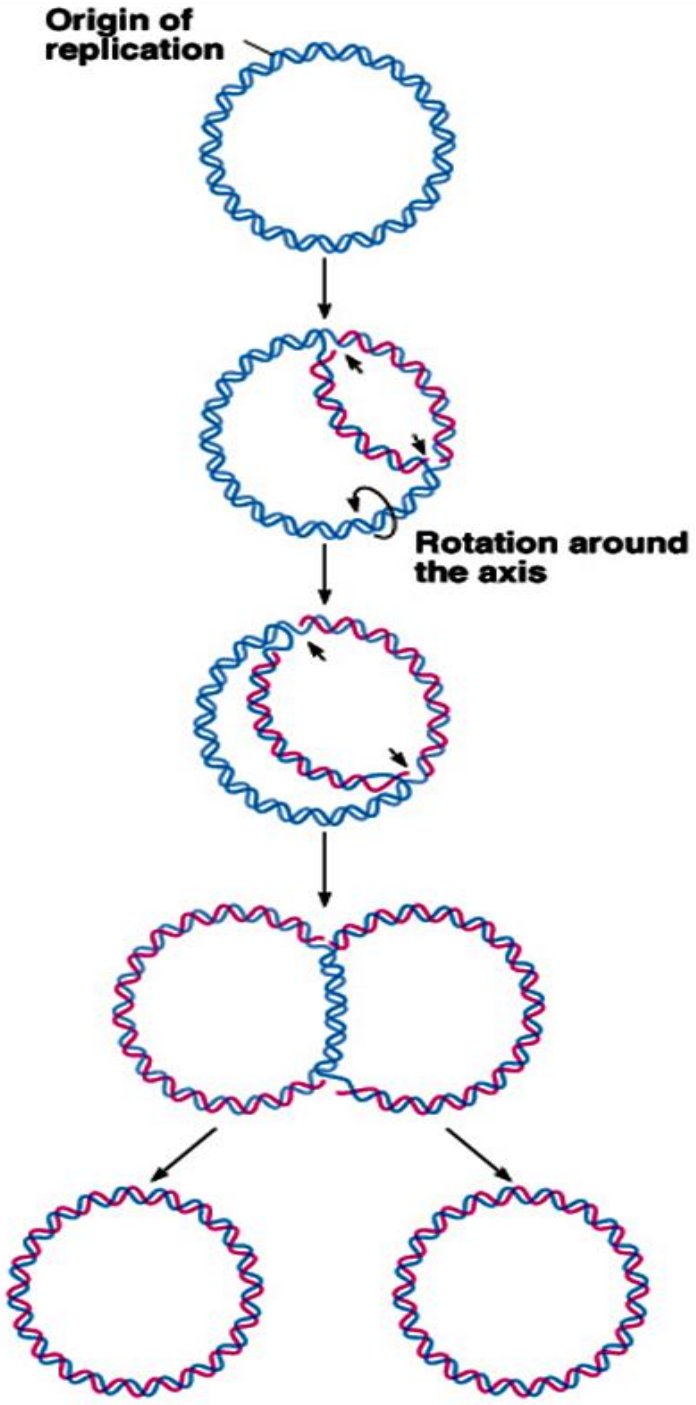

Counter-
colckwise
fork
Clockwise
fork
Completion of
Replication
Catenated Chromosome
DNA topoisomerase IV
Sepatated Chromosomes



θ replication or Cairns replication:

John Cairns allowed *E. coli* bacteria to grow on a medium containing [^{3}H] thymine so that the entire DNA of the *E. coli* is radio labeled.

The DNA was isolated carefully without fragmentation and placed on photographic film. Each ^{3}H decay exposed one grain in the film and after several months there were enough grains to visualize the molecule under microscope.

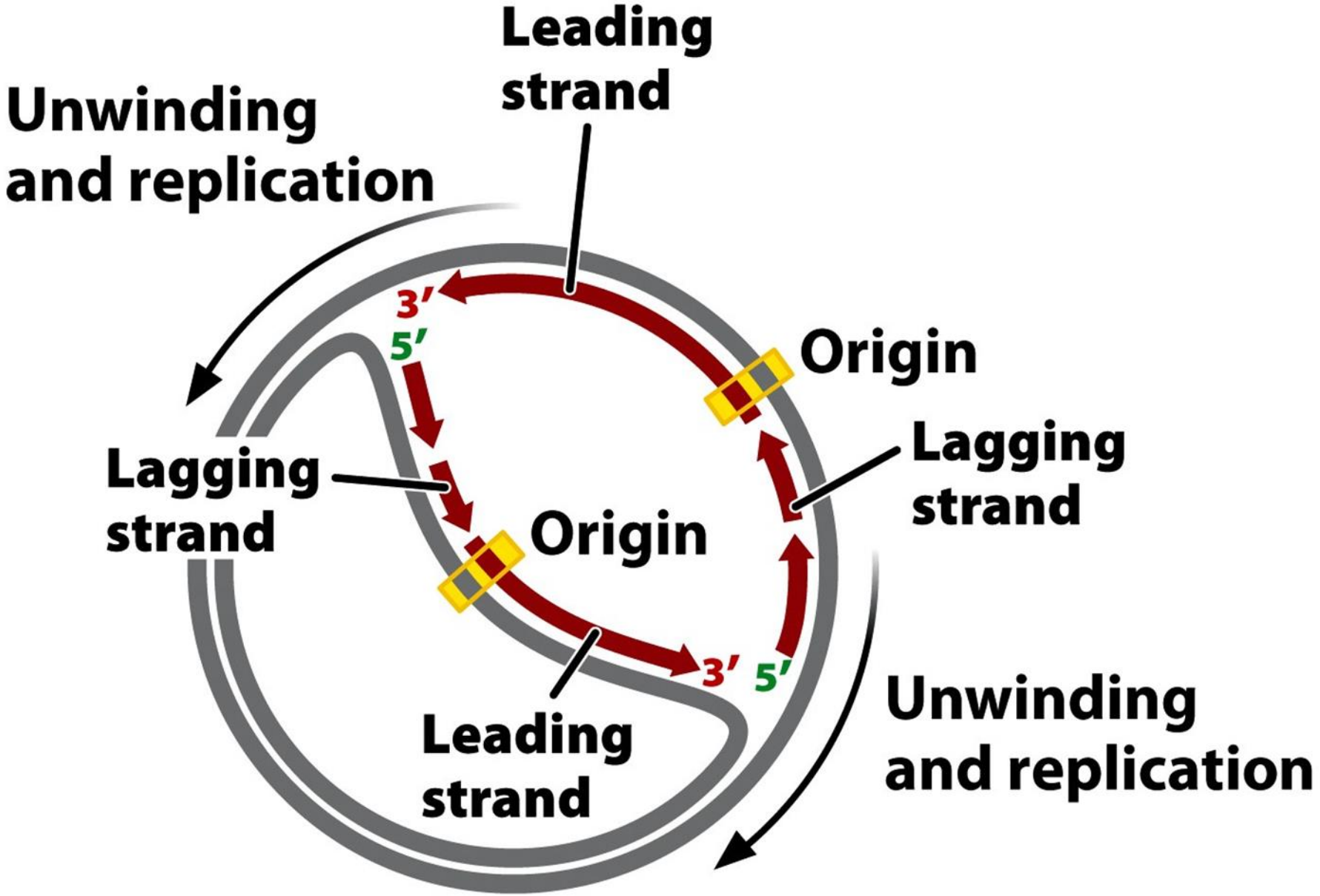


θ replication or Cairns replication:

The electron micrographs of replicating circular molecules represent the Greek letter that theta (θ) and hence this mode of replication is known as θ replication.

It is also known as Cairns replication. In this replication the polymerization may be unidirectional or bidirectional.

Theta replication or Cairns replication



Sigma replication or Rolling Circle replication or σ replication:

This type of replication is seen in bacteria (during conjugation) and phages.

In the double stranded DNA which is circular, a nick is created.

Nick has a 5` monophosphate and 3` OH terminus.

Under the influence of a helicase and SSB protein a replication fork can be generated.

No primer is required since nick has a free 3` OH group and the leading strand synthesis can proceed by elongation from this terminus.

The parental strand is displaced, at the same time it is used as a template for lagging strand synthesis.

Sigma replication or Rolling Circle replication or σ replication:

The displaced parental strand acts as a template and a new strand is synthesized in the form of short fragments which finally unite.

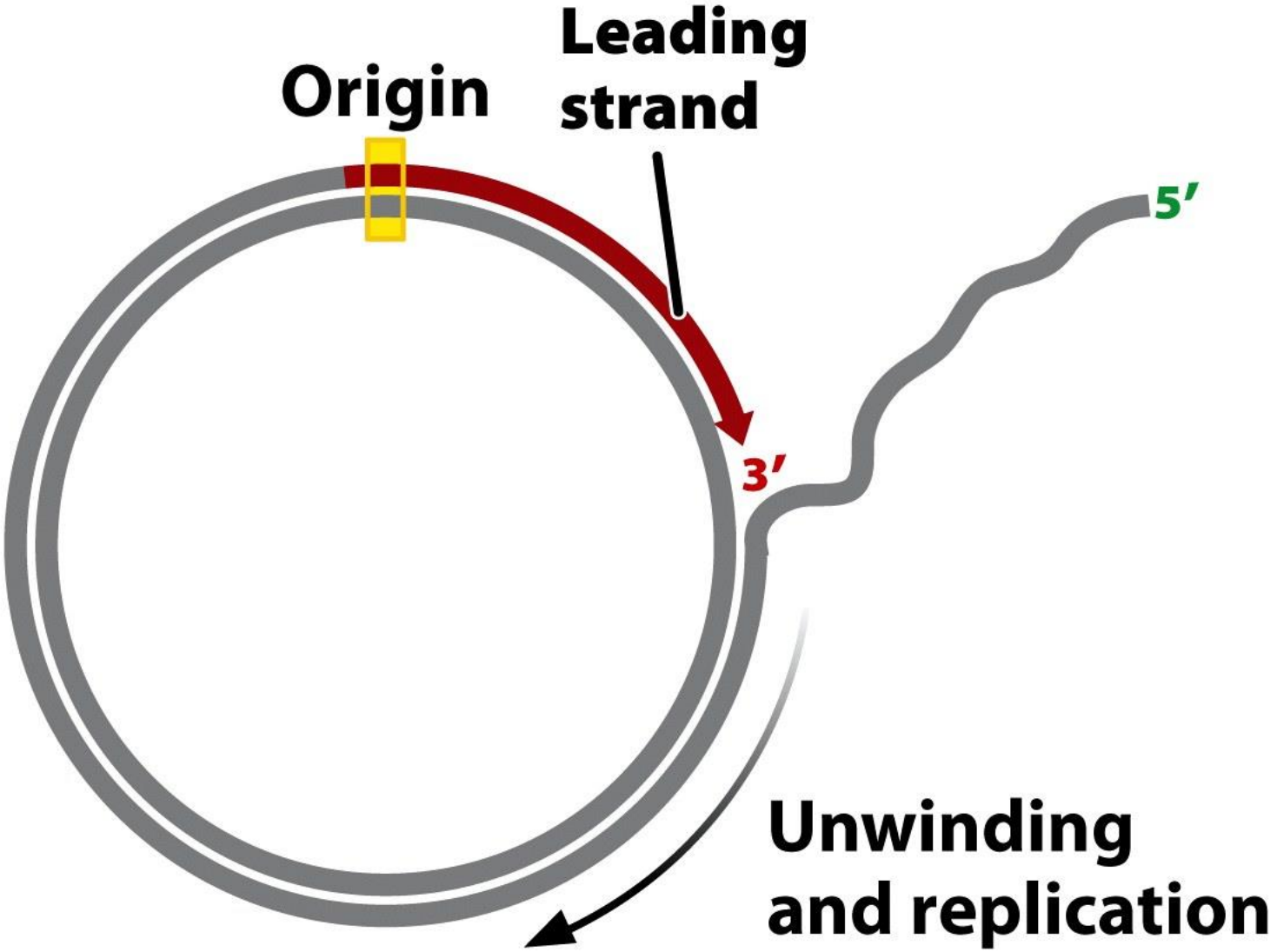
The result of such replication, a circle with linear branch is developed and as it represents the Greek letter σ, sigma it is called as σ replication or rolling circle replication.

Sigma replication or Rolling Circle replication or σ replication:

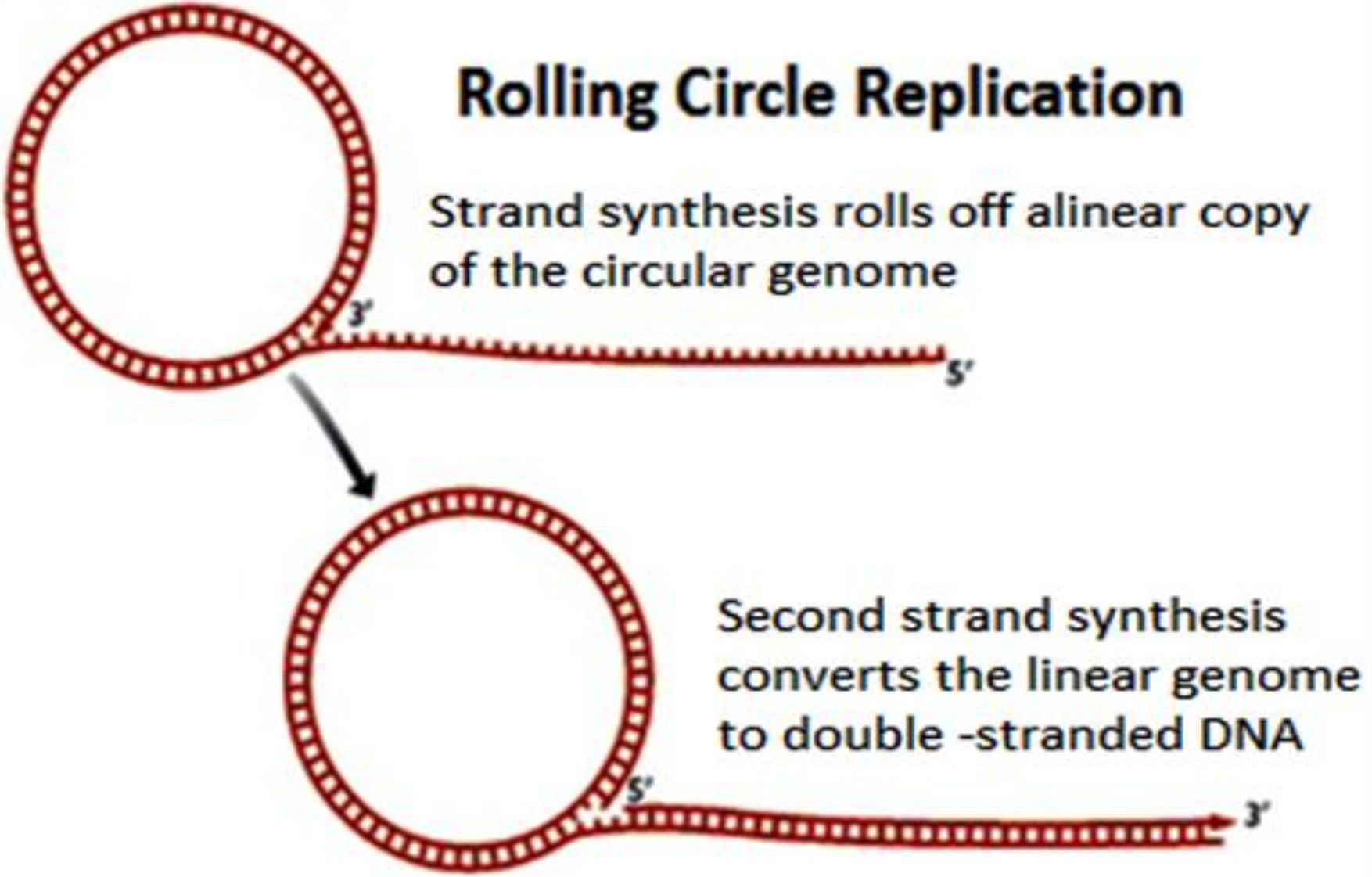
Four significant features of σ replication:

1) Leading strand is covalently linked to the parental template for the lagging strand.
2) Before precursor fragment synthesis begins the linear branch has a free 5` P terminus.
3) Rolling circle replication produces a continuous DNA strand. In phages generally a concatemeric branch (concatemeric DNA consists of unit genomes repeated in tandem) is formed by this.
4) The circular template for leading strand synthesis never leaves the circular part of the molecule.

Rolling-circle model



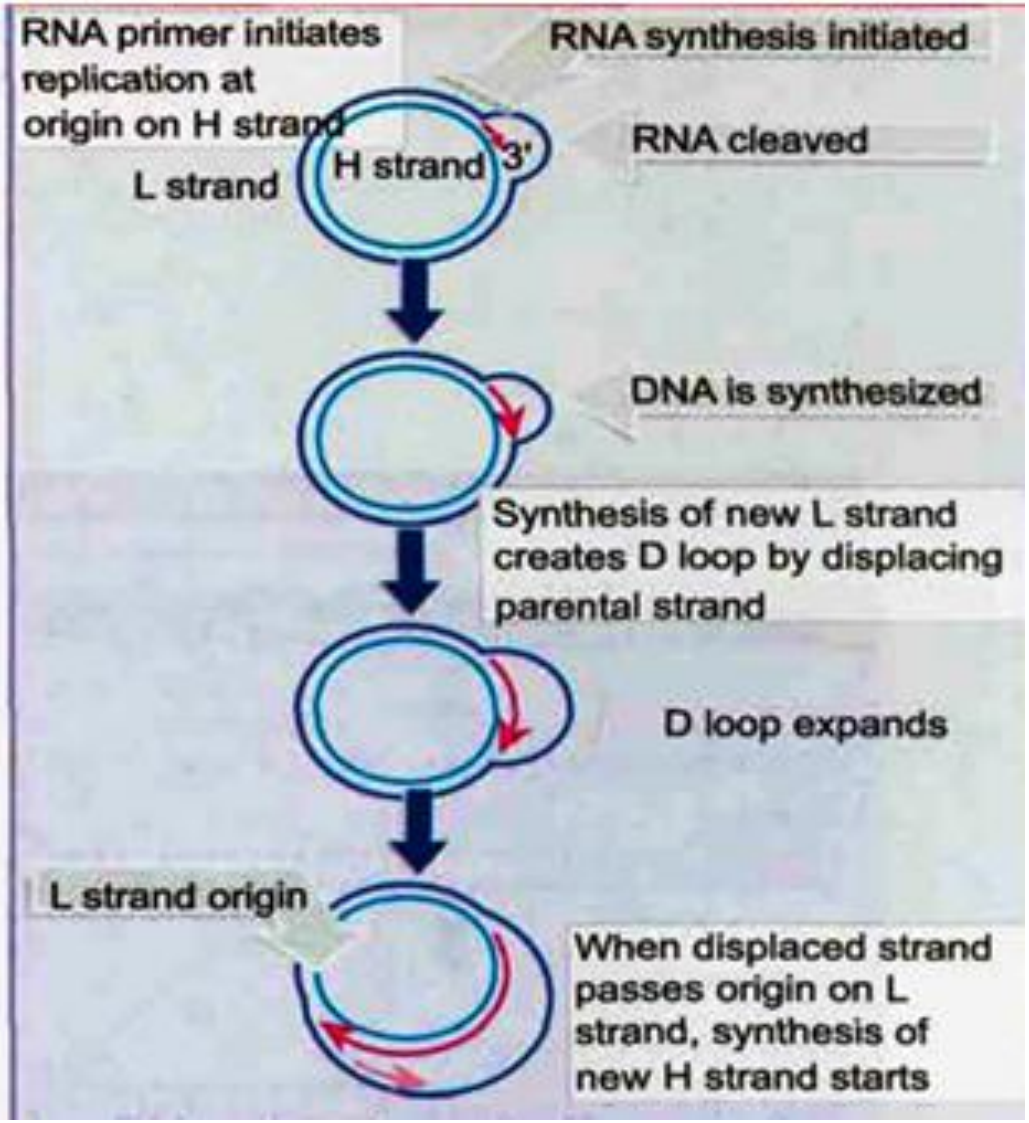
Rolling Circle Replication



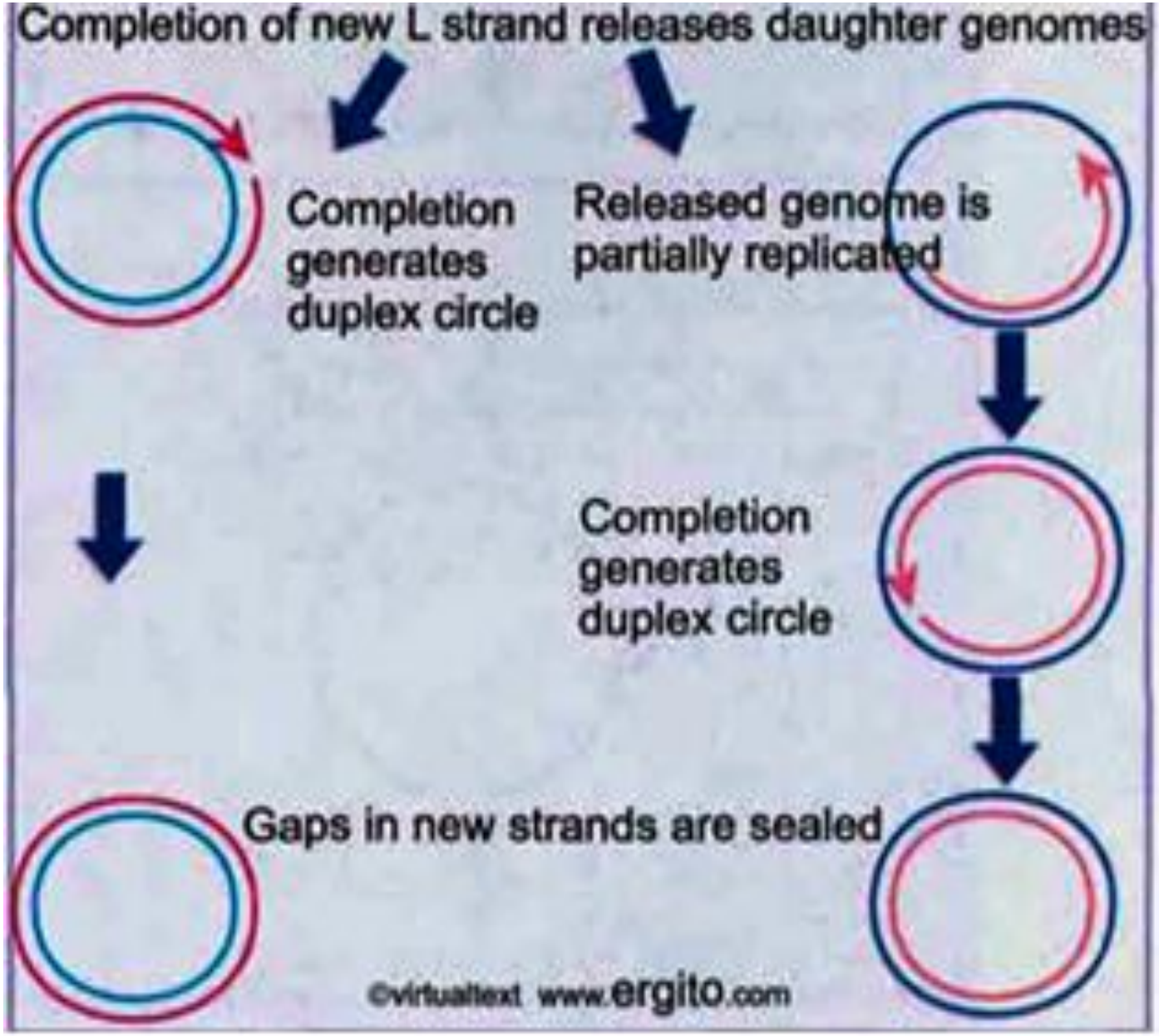
D loop or Displacement loop:

- In mitochondria the replication starts at specific origin in the circular DNA molecule.
- But only one of the 2 parental strands (the H strand in mammalian mitochondria DNA) is used as a template for the synthesis of new strand.
- The synthesis proceeds for only a short distance displacing the parental (L) strand that remains single stranded.
- This condition is known as displacement loop or D loop.
- A single D loop is found as an opening of 500-600 bases in mammalian mitochondria.

D loop


RNA primer initiates replication at origin on H strand
RNA synthesis initiated
H strand
3'
L strand
RNA cleaved
DNA is synthesized
Synthesis of new L strand creates D loop by displacing parental strand
D loop expands
L strand origin
When displaced strand passes origin on L strand, synthesis of new H strand starts

Replication in Mitochondria

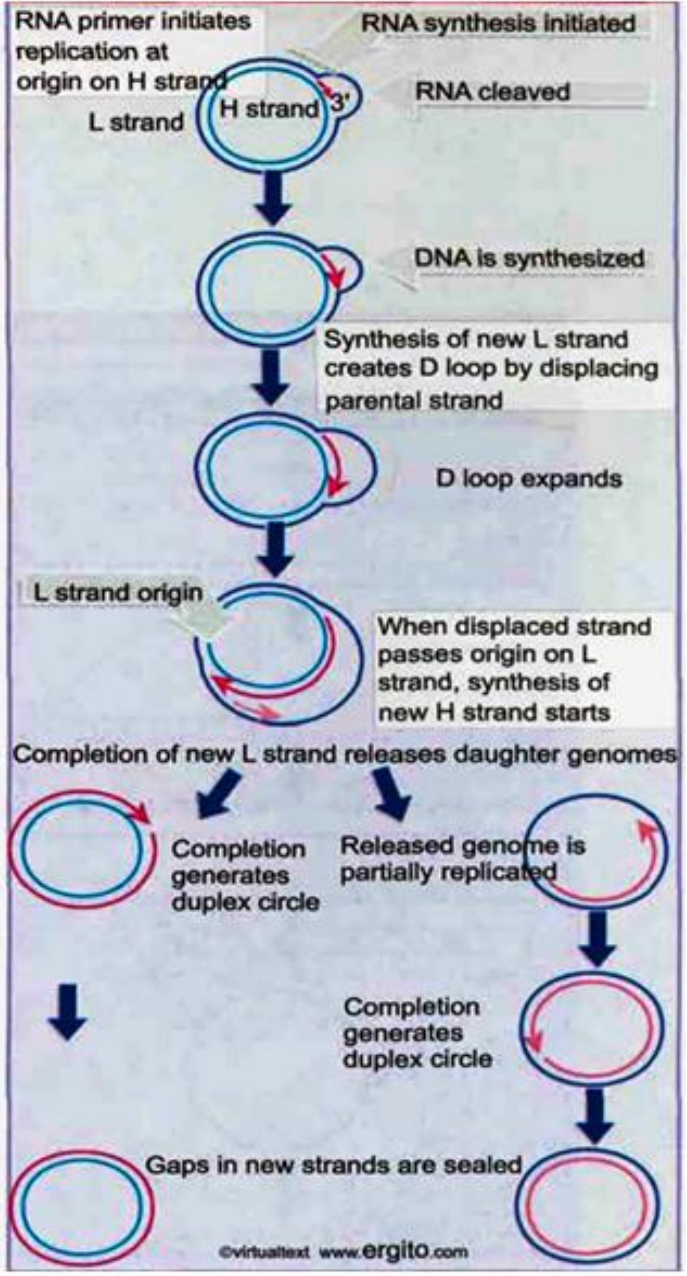


D loop or Displacement loop:

- The short single strand that maintains D loop is unstable and turns over.
- It is frequently degraded and re-synthesized to maintain the opening of the duplex in this site.
- In some mitochondria several D loops are seen.
- D loops are also seen in chloroplasts.
- To replicate the mitochondrial DNA the short strand in the D loop is extended.
- The displaced region of the original strand becomes longer expanding the D loop.
- This D loop expands till it reaches a point about 2/3 of the way round the circle.

D loop or Displacement loop:

- Replication in this region exposes the origin in the second strand.
- Using this new H strand will be synthesized by extending the primer synthesized by primase as in the first case.
- Due to the lag in the start of the H strand synthesis the L strand synthesis is completed first there by generating one complete duplex circle and one gapped circle, which will be later converted a duplex circle.
- Finally the 2 strands are sealed to become covalently intact.
- From the study of D loops of replication it may be concluded that “The opening of the duplex does not necessarily lead to the initiation of replication in the other strand.
- In mitochondria the origins for replication lie at different locations in complementary strands”.



Mitochondrial DNA showing D loop during replication

Replication in Eukaryotes

Eukaryotic polymerases

- At least 15 DNA Polymerases are seen in eukaryotes. They include Pol α/primase, Pol β, Pol γ, Pol δ, Pol ε, η, ι, κ, Rev1, Pol ζ ,θ, λ, ϕ, σ, and μ.
- All polymerases exhibit 5→3 polymerization.
- None of the eukaryotic polymerases can remove primers.
- Only the polymerases that deal with the elongation (γ, δ and ε) have proofreading ability.

Eukaryotic polymerases

- **Pol α/primase** (DNA primase, **RNA** polymerase)- a tetrameric protein, Located in nucleus, exhibits both primase and DNA Polymerase activities.
- Tetramer – 4 subunits POLA 1 (catalytic) POLA 1 (regulatory) POLA 3,4 (Primase) - larger subunit - 5´-3´ polymerization activity -Two smaller subunit – primase activity - one subunit – assist in other three subunits.
- Primase subunit synthesizes a short primer (8-16 nucleotides long RNA), extended by polymerase subunit & 10-20 nucleotides long DNA (iDNA) is formed.

Eukaryotic polymerases

- After Primer synthesis DNA polymerase α will be replaced by DNA polymerase ε (on the leading strand) or DNA polymerase δ (on the lagging strand) which carry out elongation.
- **Pol δ**: Has high processivity and 3'→5' exonuclease activity. Synthesizes lagging strand.
- **Proliferating Cell Nuclear Antigen (PCNA) -** a *homotrimeric protein* increases processivity of DNA polymerase **δ,** similar to β clamp of DNA Pol III.
- **The replication factor C (RFC)** is a clamp loader, similar to the γ Complex in *E. coli*. It uses ATP hydrolysis to open the ring of PCNA and loads it on 3' end of DNA.

The replication factor C (RFC)

- The replication factor C, or RFC, a **5**-subunit protein complex, is a clamp loader, similar to γ Complex of DNA pol III in *E. coli*.
- The subunits of this heteropentamer are named Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5 (in *S. cerevisiae*).
- RFC It loads PCNA on to DNA using ATP hydrolysis so that PCNA can encircle the DNA.

Eukaryotic polymerases

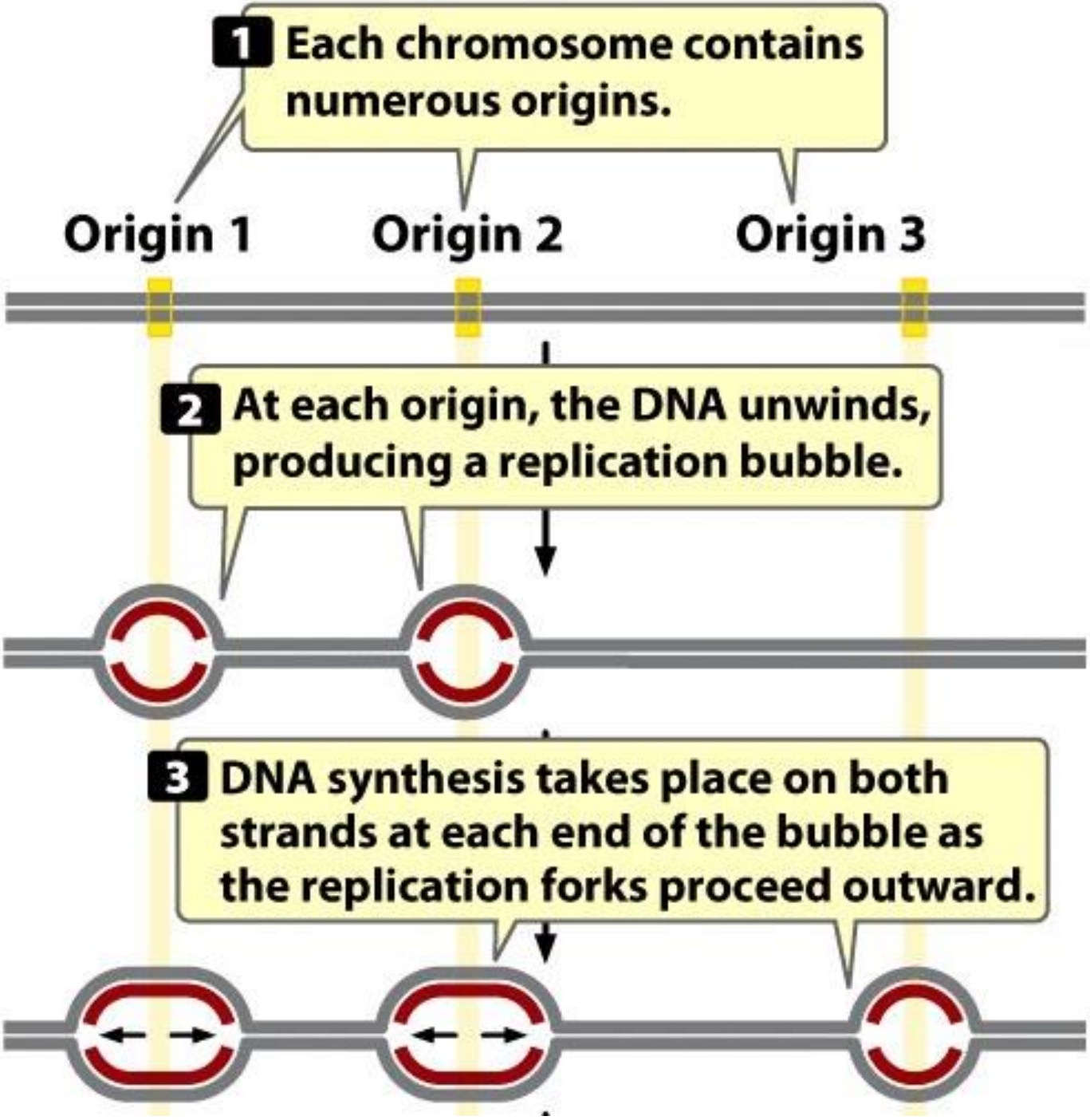
- **Pol ε**: Has high processivity and 3'→5' exonuclease activity. Synthesizes leading strand & is related to pol δ.
- **Pol γ**: Replicates mitochondrial DNA. It is similar to bacterial DNA polymerase and has proofreading 3'→5' exonuclease activity.
- **Pol β**: Useful in base excision repair and gap-filling synthesis.
- The other eukaryotic polymerases are not very well characterized.

- **Origin Recognition Complex** or **ORC** (Orc1-6) binds to origins of replication in ATP-dependent manner, similar to DnaA protein in *E. coli*.
- It is similar to Dna A protein and binds to origin of replication to initiate replication.
- **Mini Chromosome Maintenance complex**, or Mini Chromosome Maintenance (MCM) protein complex is a helicase similar to DnaB protein of *E. coli*.
- MCM is also a hexamer of six related polypeptides (Mcm2-7).

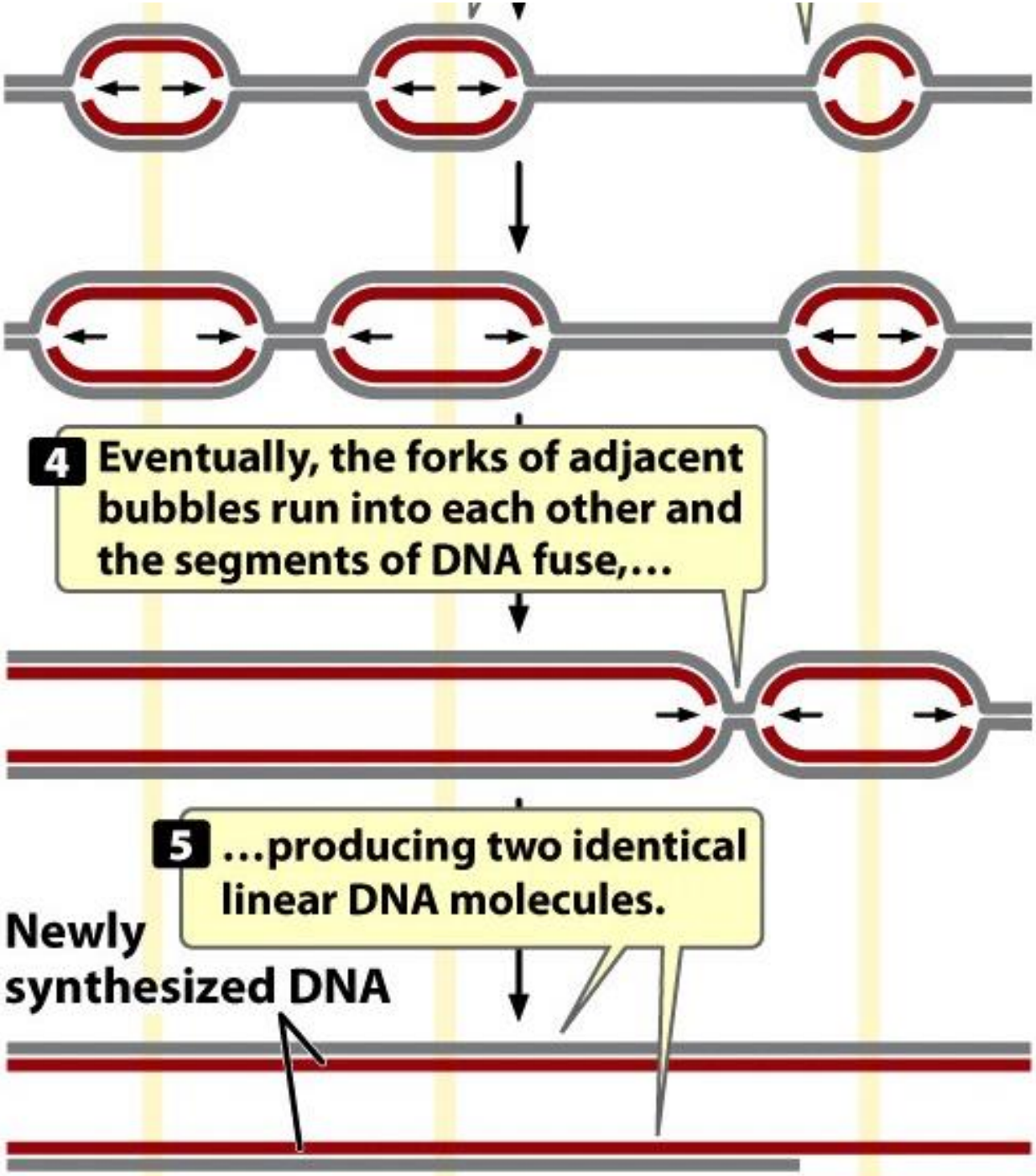
- **Replication protein A (RPA)** is a similar to SSB protein of prokaryotes.
- Replication protein A (RPA) is a heterotrimeric protein composed of 70, 34, and 14kDa subunits.
- Functions of SSB protein and RPA are similar.

An autonomously replicating sequence (ARS) or Replicator

- An autonomously replicating sequence (ARS) contains the origin of replication in the yeast and lower eukaryotes.
- It contains four regions (A, B1, B2, and B3) & when these regions are mutated, replication does not initiate.
- In higher eukaryotes the size of such sequences vary from 500 - 5000bp.

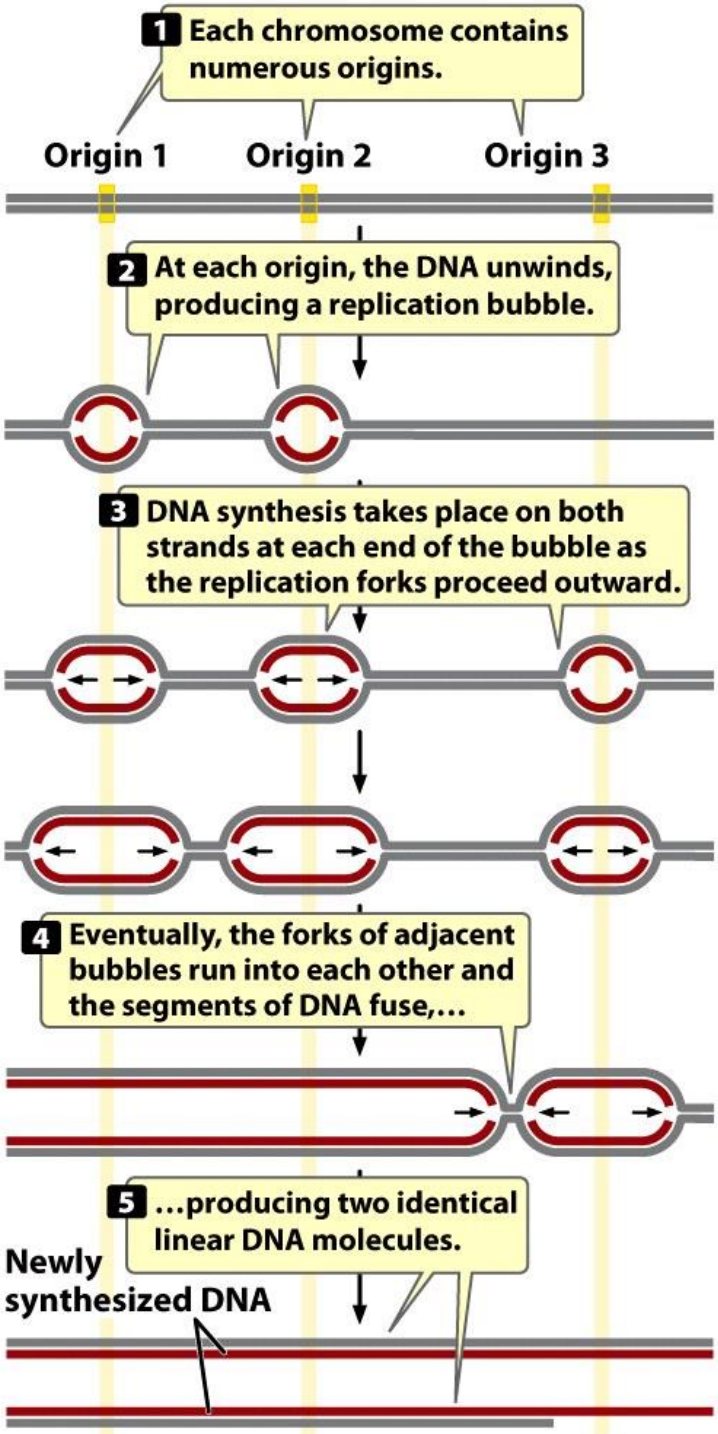


Eukaryote Replication



Eukaryote Replication

Eukaryote Replication



Conclusion: The products of eukaryotic DNA replication are two linear DNA molecules.

- **Geminin** (25 kD) a nuclear protein of about 200 amino acids, is an **inhibitor** of DNA replication and substrate of the anaphase promoting complex (APC).
- Note: **Anaphase-Promoting Complex**, also called cyclosome (**APC/C**), is a large complex of 11–13 subunits, identifies or marks cell cycle proteins to be degraded by 26S proteasome.

- **Licensing Factor** is a protein or complex of proteins that allows an origin of replication to begin DNA replication.
- These factors are found in different places in different organisms. Ex. In metazoan organisms, they are commonly synthesized in the **cytoplasm** of the cell to be imported into the **nucleus** when required.
- **Cdc6 and MCM** proteins provide the licensing function In **yeast**.

Eukaryotic Replication

- Initiation
- Elongation and
- Termination.

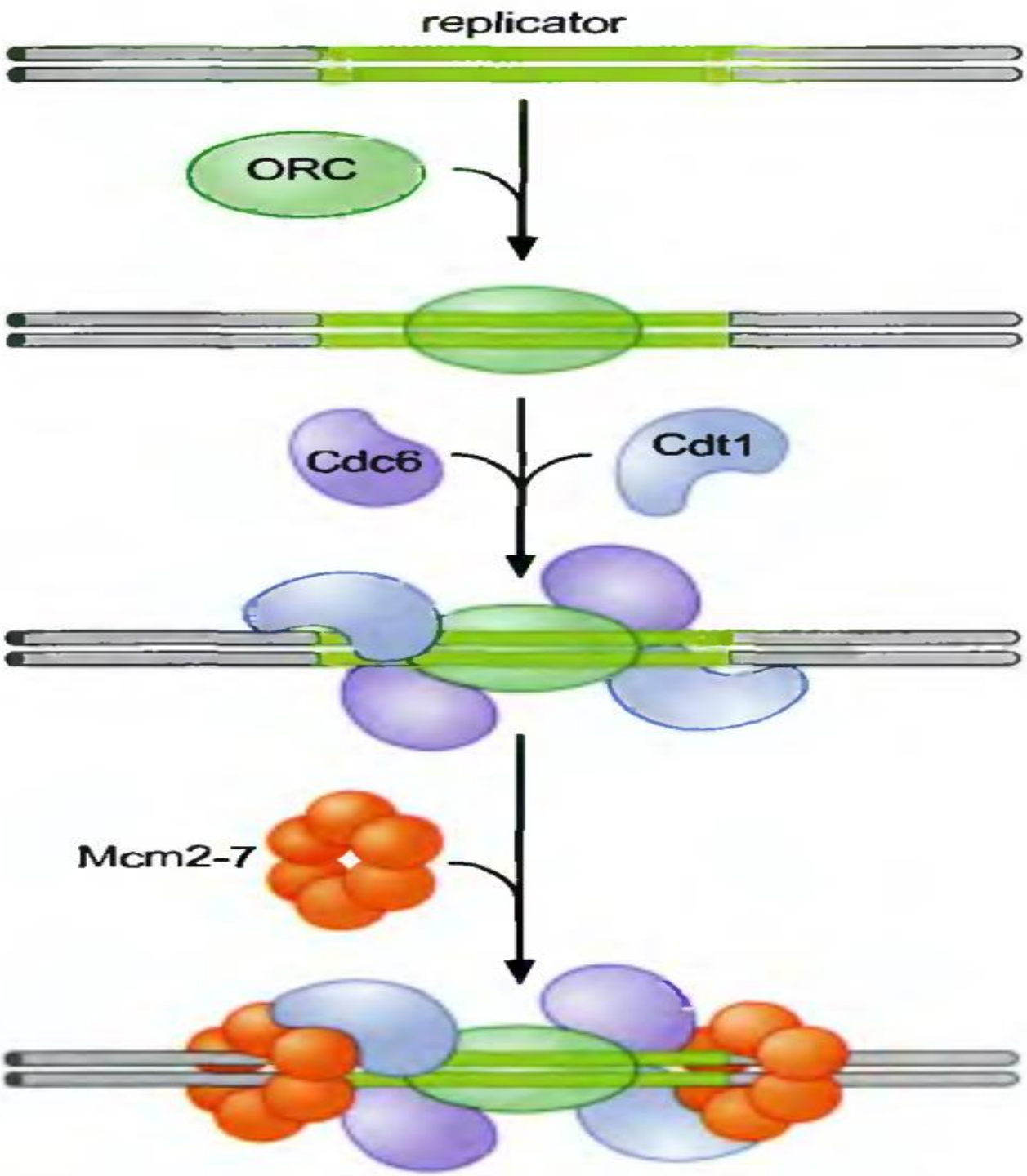
Initiation of eukaryotic replication

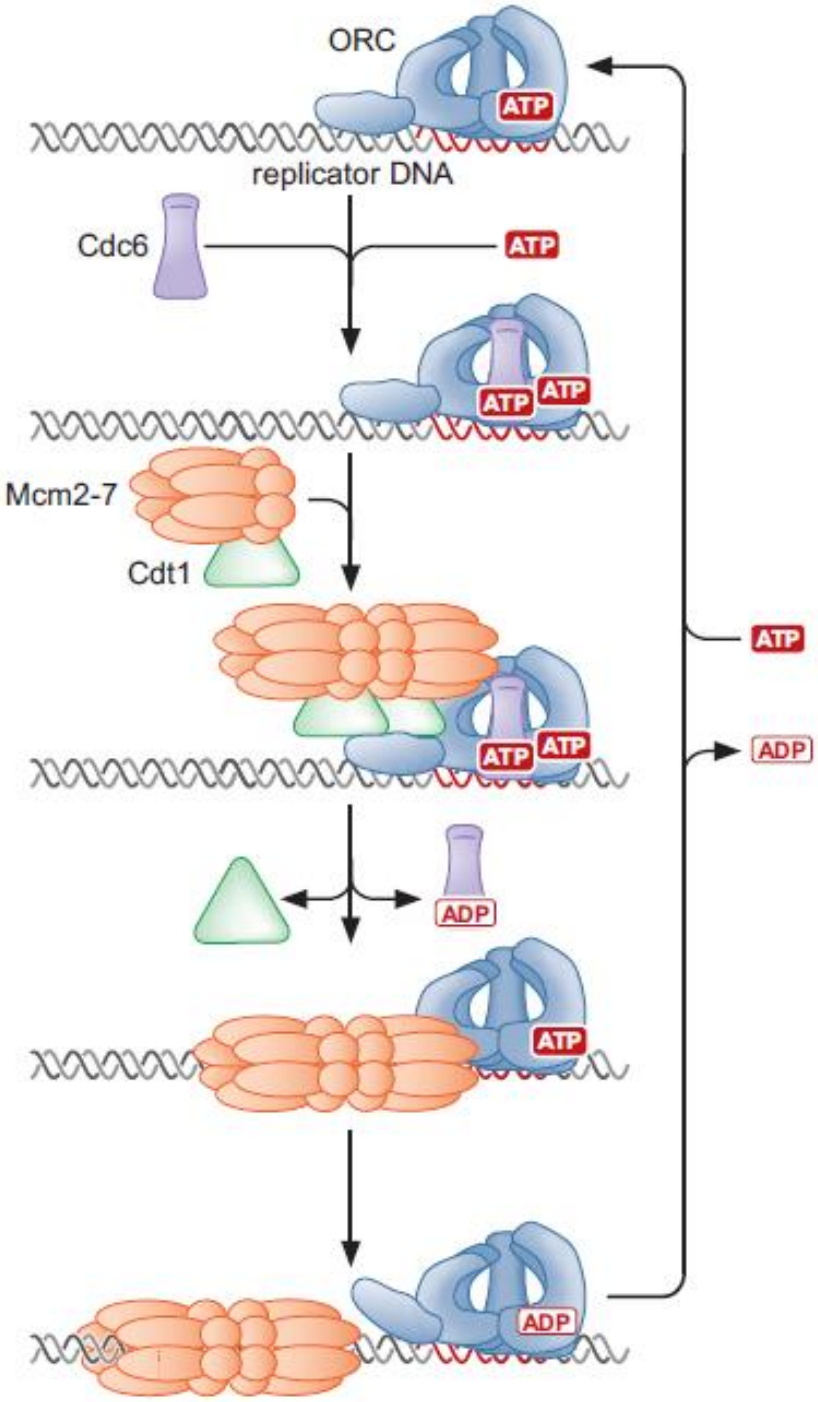
- **The initiation** requires two steps to occur at distinct times in the cell cycle –

  (1) Replicator selection

  (2) Origin activation.

Replicator selection - Formation of the Pre-Replicative Complex (Pre-RC)





Eukaryotic helicase loading.

- Initially the ATP-bound origin recognition complex (ORC) joins with the replicator.
- ORC recruits ATP-bound Cdc6 and two copies of the Mcm2-7 helicase bound to a second helicase loading protein, Cdt1.
- This assembly of proteins triggers ATP hydrolysis by Cdc6, resulting in the loading of a head-to-head dimer of the Mcm2-7 complex encircling double-stranded origin DNA and the release of Cdc6 and Cdt1 from the origin.
- Subsequent ATP hydrolysis by ORC is required to reset the process (illustrated as release from Mcm2-7). Exchange of ATP for ADP allows a new round of helicase loading.

Replicator selection

- Occurs in G1 Phase (prior to S phase).
- It leads to the assembly of a multiprotein complex - Pre-Replicative Complexes (pre-RCs) composed of 4 separate proteins (ORC, Cdc6, Cdt1 and Mcm Complex) at each replicator in the genome.

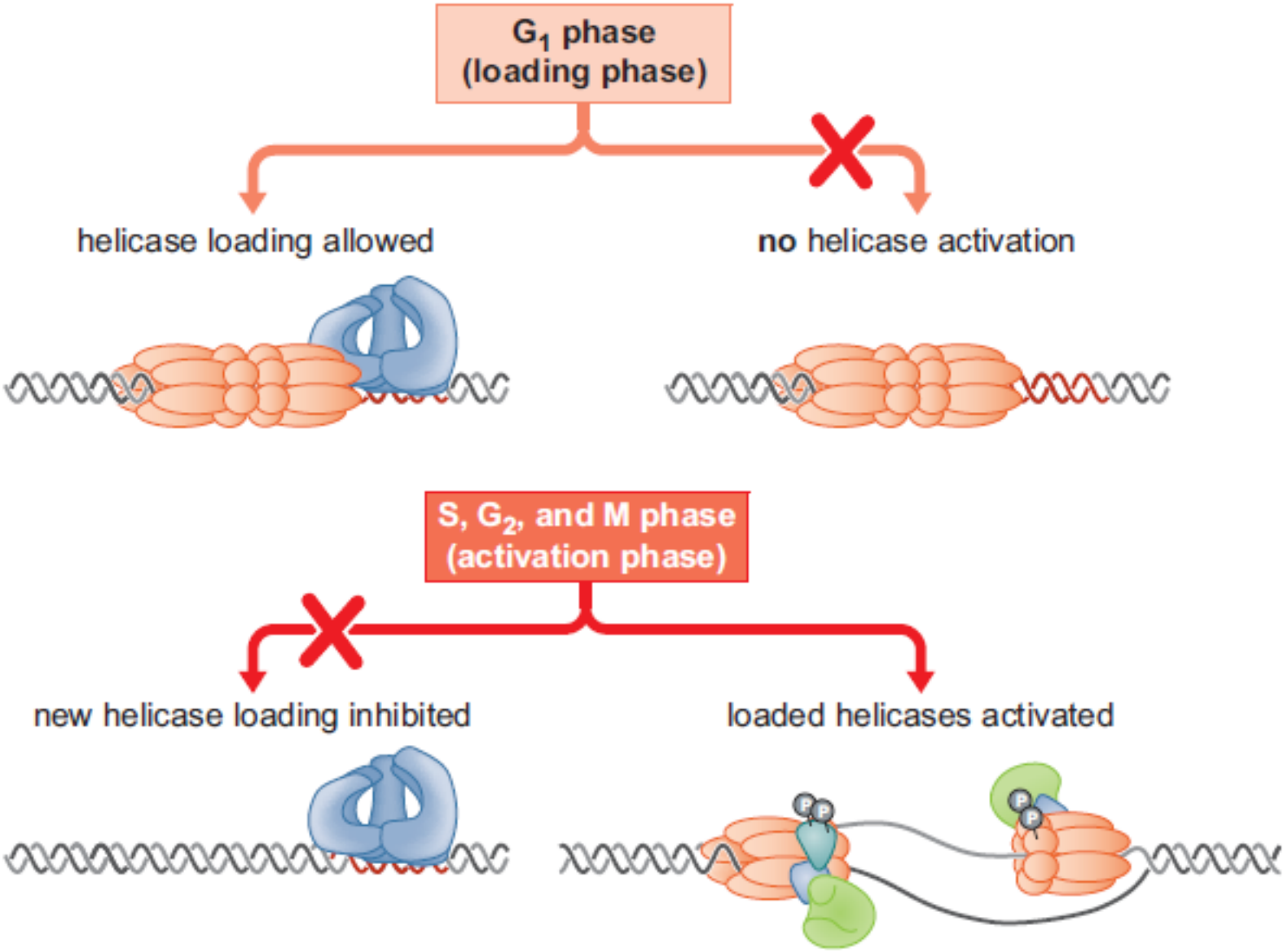
Pre-Replicative Complex Formation

- ORC recognizes the replicator, recruits two helicase loading proteins (Cdc6 and Cdt1).
- ORC and helicase loading proteins recruit Mcm 2-7 complex (eukaryotic replication fork helicase) leading to the formation of the Pre-Replicative Complex.
- Pre-RC formation does not lead to the immediate unwinding of origin DNA or the recruitment of DNA polymerases.
- The pre-RCs formed during G1 are activated to initiate replication only after cells pass from G1 to S phase of the cell cycle.

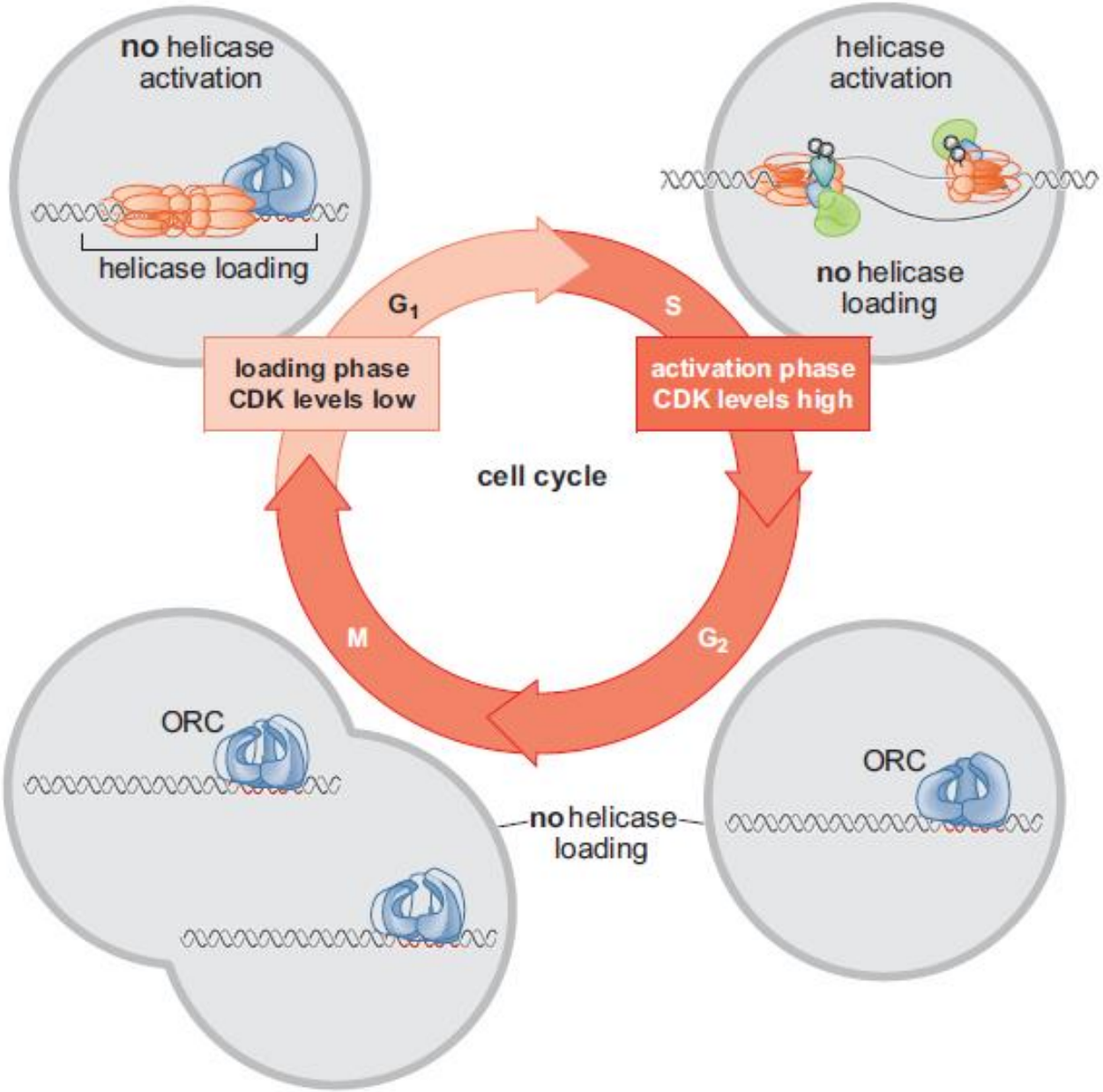
- **Origin activation** only occurs after cells enter S phase and triggers the replicator-associated protein complex to initiate DNA unwinding and DNA polymerase recruitment.

- Pre-RCs are activated by 2 protein kinases (Cdk and Ddk) which are inactive in G1 (where the Cdk levels are low).
- Cdk is activated only when cells enter S phase (where the Cdk levels are high).
- Once activated, these kinases target the Pre-RC and other replication proteins.
- Phosphorylation of these proteins results in the assembly of additional replication proteins including the three eukaryote DNA polymerases and a number of other proteins required for their recruitment at the origin and the initiation of replication.

Eukaryotic helicase loading and activation occur during different cell cycle stages.



Cell cycle regulation of CDK activity controls replication



- DNA Pol δ and ε associate first at the replication fork, followed by DNA Pol α/primase.
- This order ensures that all three DNA polymerases are present at the origin prior to the synthesis of the first RNA primer (by DNA Pol α/primase).
- Only a subset of the proteins that assemble at the origin go on to function as part of the eukaryote replisome.

- In addition to the three DNA polymerases, the Mcm complex and many of the factors required for DNA polymerase recruitment become part of the replication fork machinery.
- Similar to the *E. coli* DNA helicase loader (DnaC), the other factors (such as Cdc6 and Cdt1) are released or destroyed after their role is complete.

- The tight connection between pre-RC function, cyclin-dependent kinases (Cdk) levels, and the cell cycle ensures that the eukaryotic genome is replicated only once per cell cycle.
- Cdks play 2 roles in regulating pre-RC function.

(1) Cdks activate existing pre-RCs to initiate DNA replication and

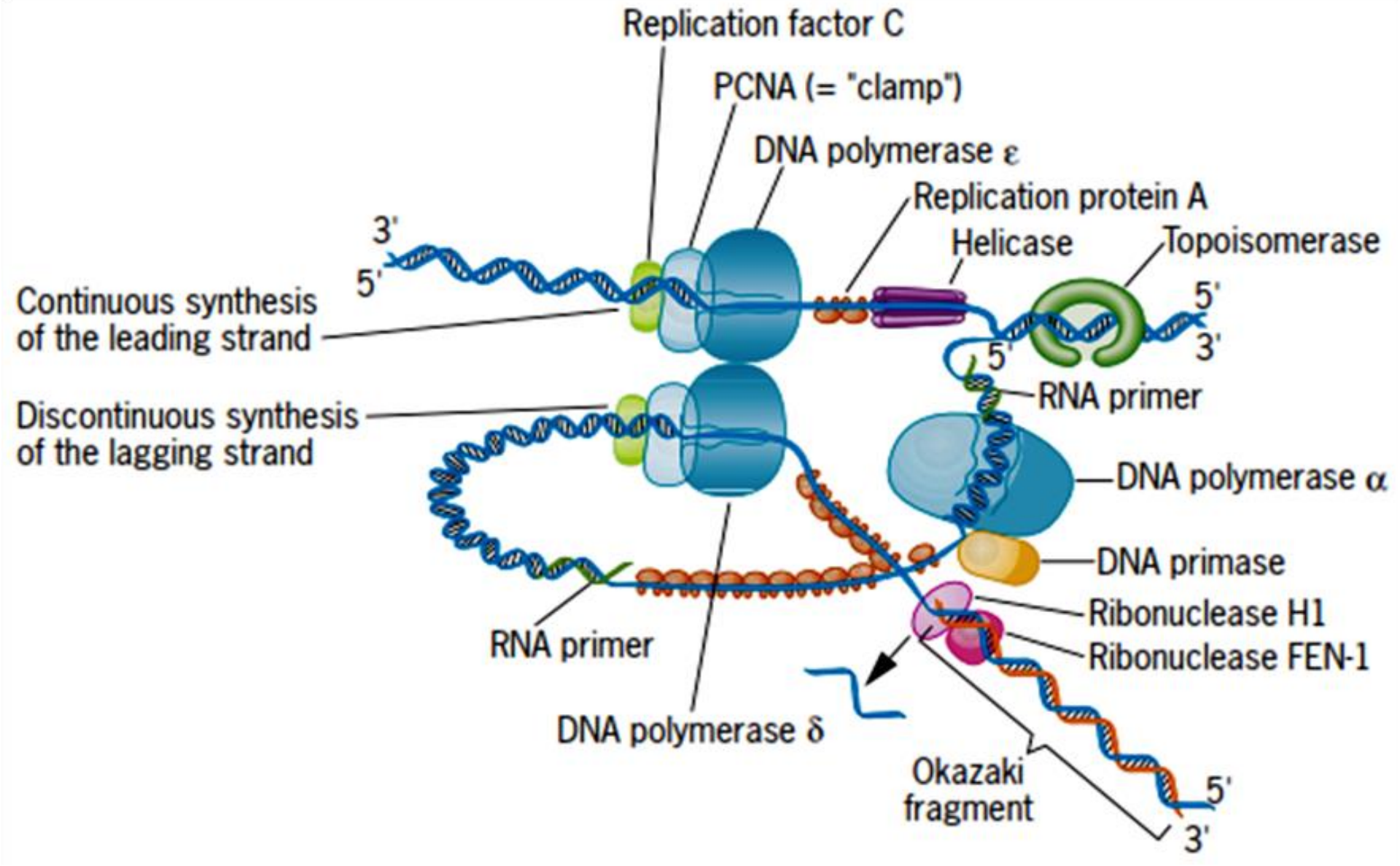
(2) Cdks inhibit the formation of new pre-RCs.

- DDK (Dbf4-dependent) and DNA repair kinases also participates in ORC activation and triggers the transition into DNA replication.

Elongation

- **Elongation** is more or less same in case of prokaryotes and eukaryotes leading and lagging strand synthesis.
- The major difference is the size of Okazaki fragments in lagging strand.
- In prokaryotes the size varies from 1000 – 2000 nucleotides where as in eukaryotes the size is in between 100 – 200 nucleotides.

Eukaryotic Replication of DNA



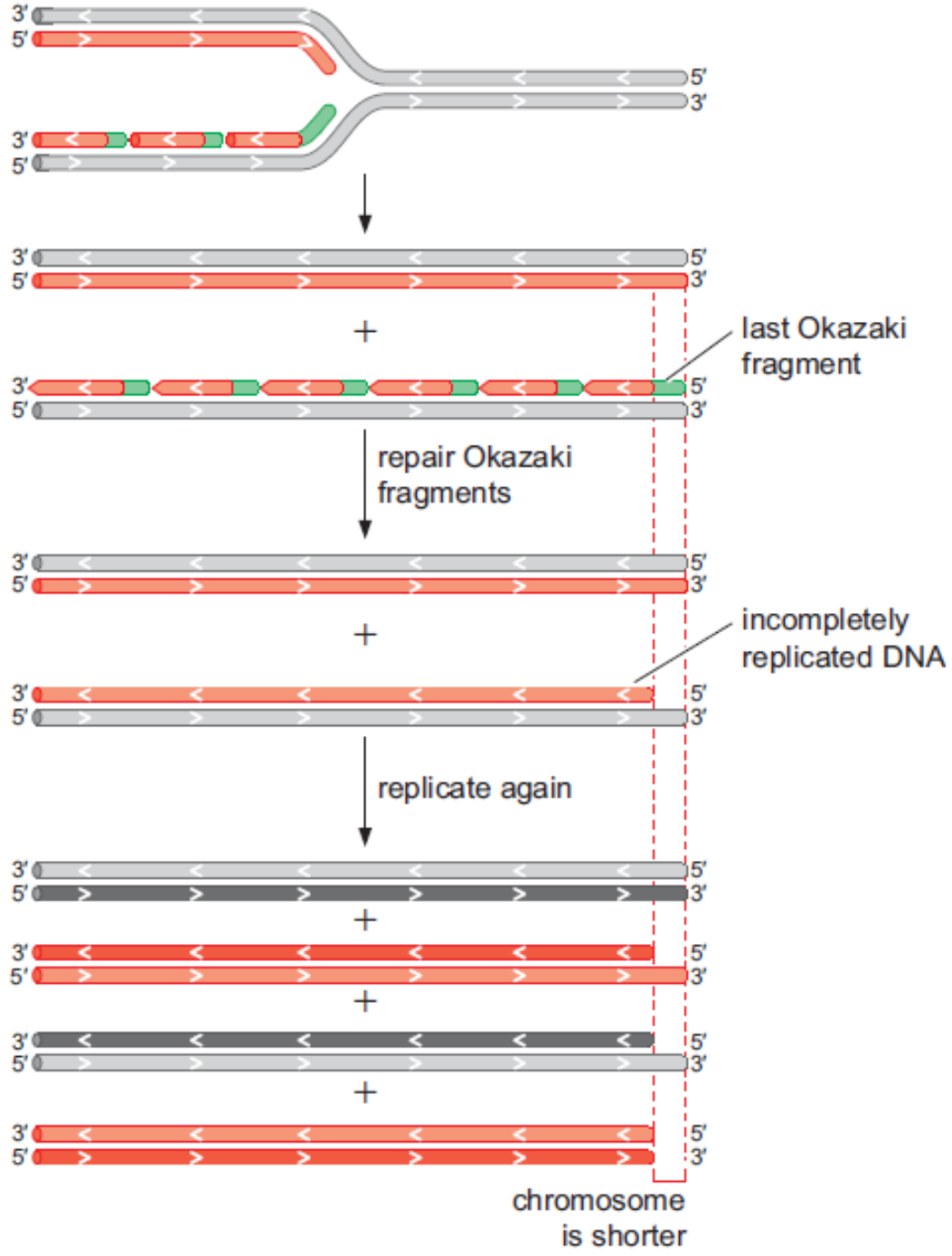
Termination

- **Termination** is also different because in eukaryotes the DNA is linear unlike in prokaryotes.
- There is no inherent topological linkage after the replication of a linear molecule, however due to the large size of eukaryotic chromosomes the DNA is folded into loops attached to a protein scaffold.
- These attachments lead to many of the same problems that circular chromosomes have when the two daughter chromosomes must be separated.
- Type II topoisomerases help in the segregation of these large linear molecules.

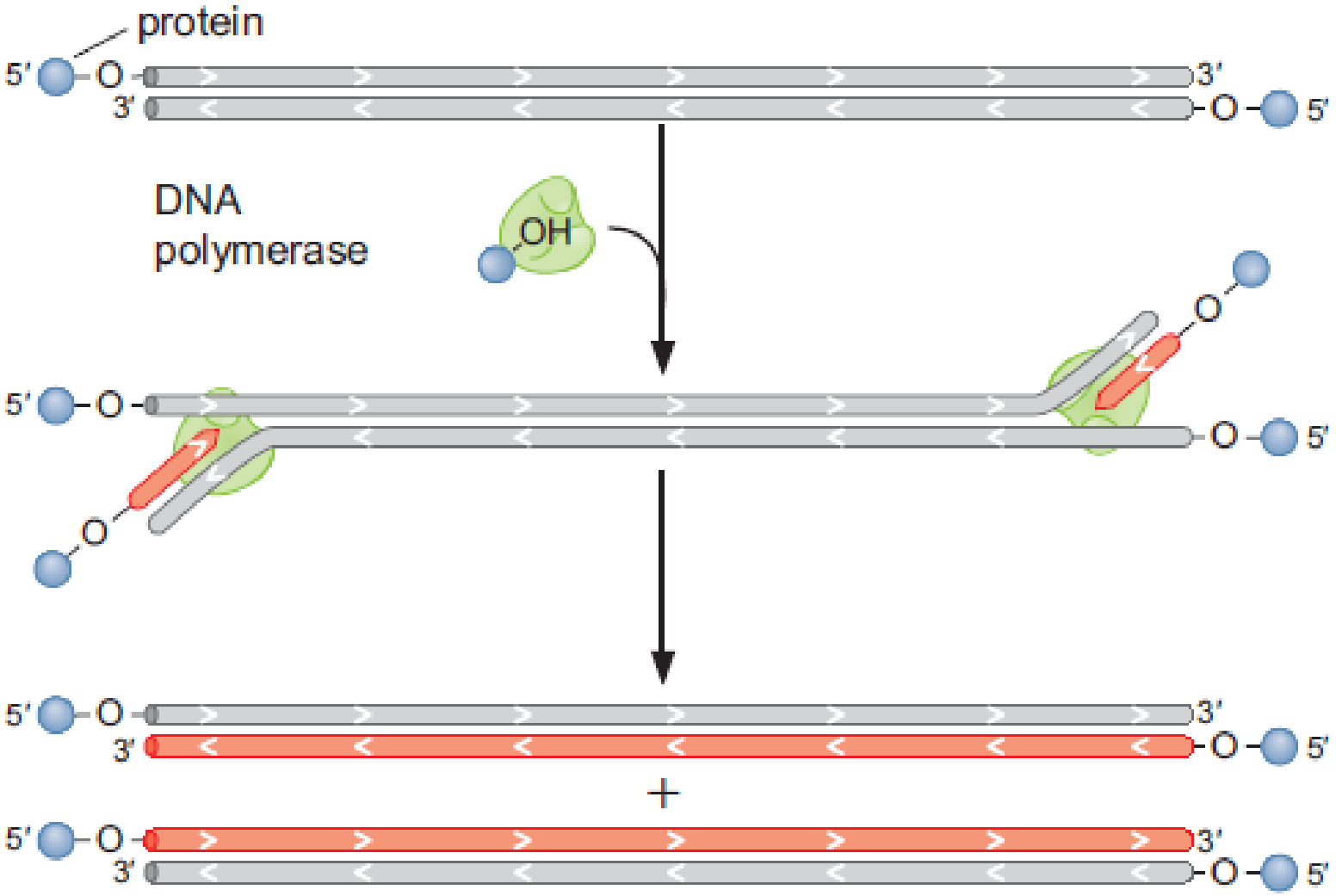
Primer removal

- To replace the RNA primers with DNA, an enzyme called RNase H recognizes and removes most portion of each RNA primer.
- This enzyme specifically degrades RNA that is base-paired with DNA (hence, the "H" in its name, which stands for hybrid in RNA: DNA hybrid).
- RNase H removes all of the RNA primer except the ribonucleotide directly linked to the DNA end. This is because RNAse H can only cleave bonds between two ribonucleotides.
- The final ribonucleotide is removed by an exonuclease FEN-1 that degrades RNA or DNA from their 5' end.

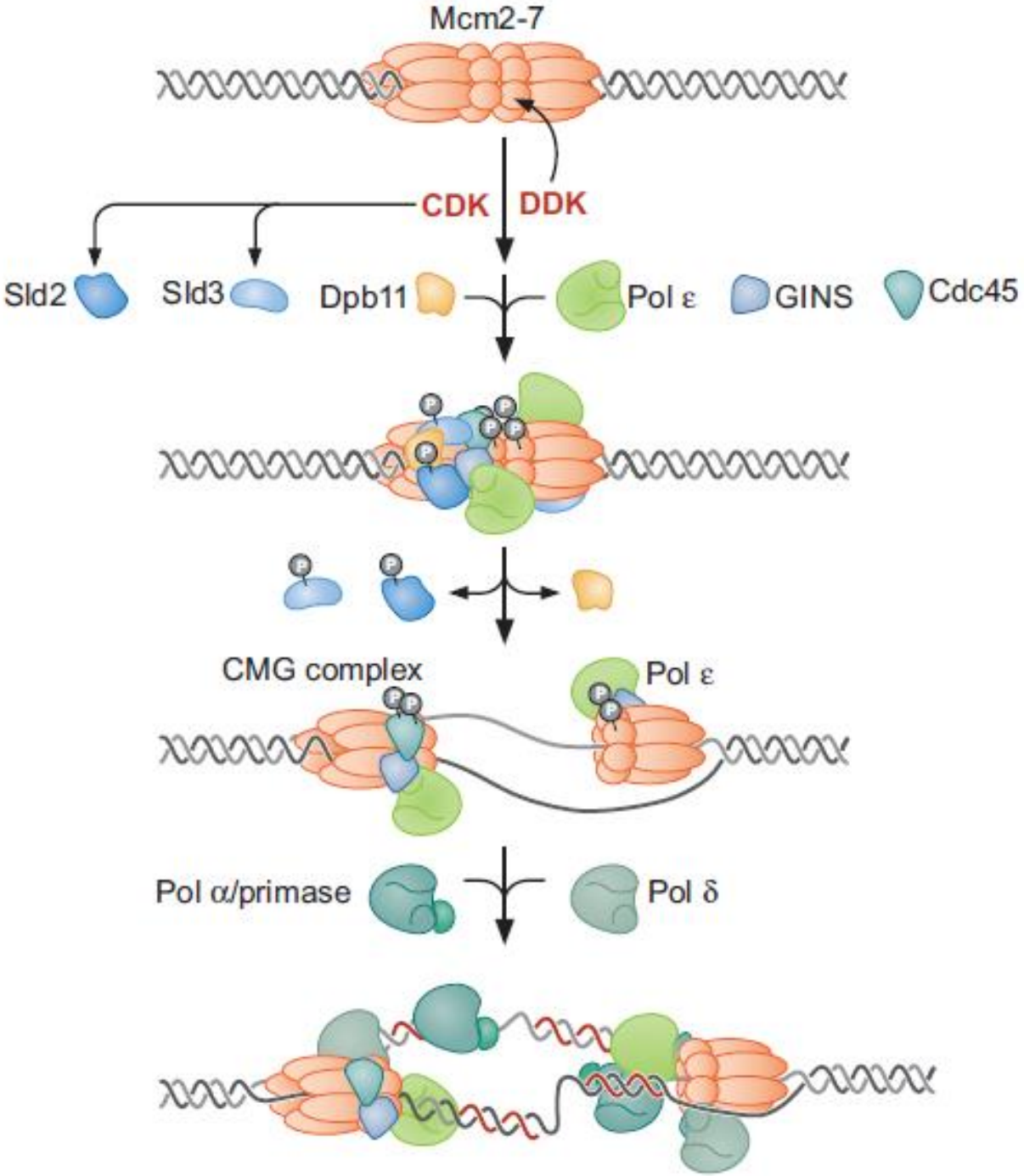
The end replication problem



Protein priming as a solution to the end replication problem



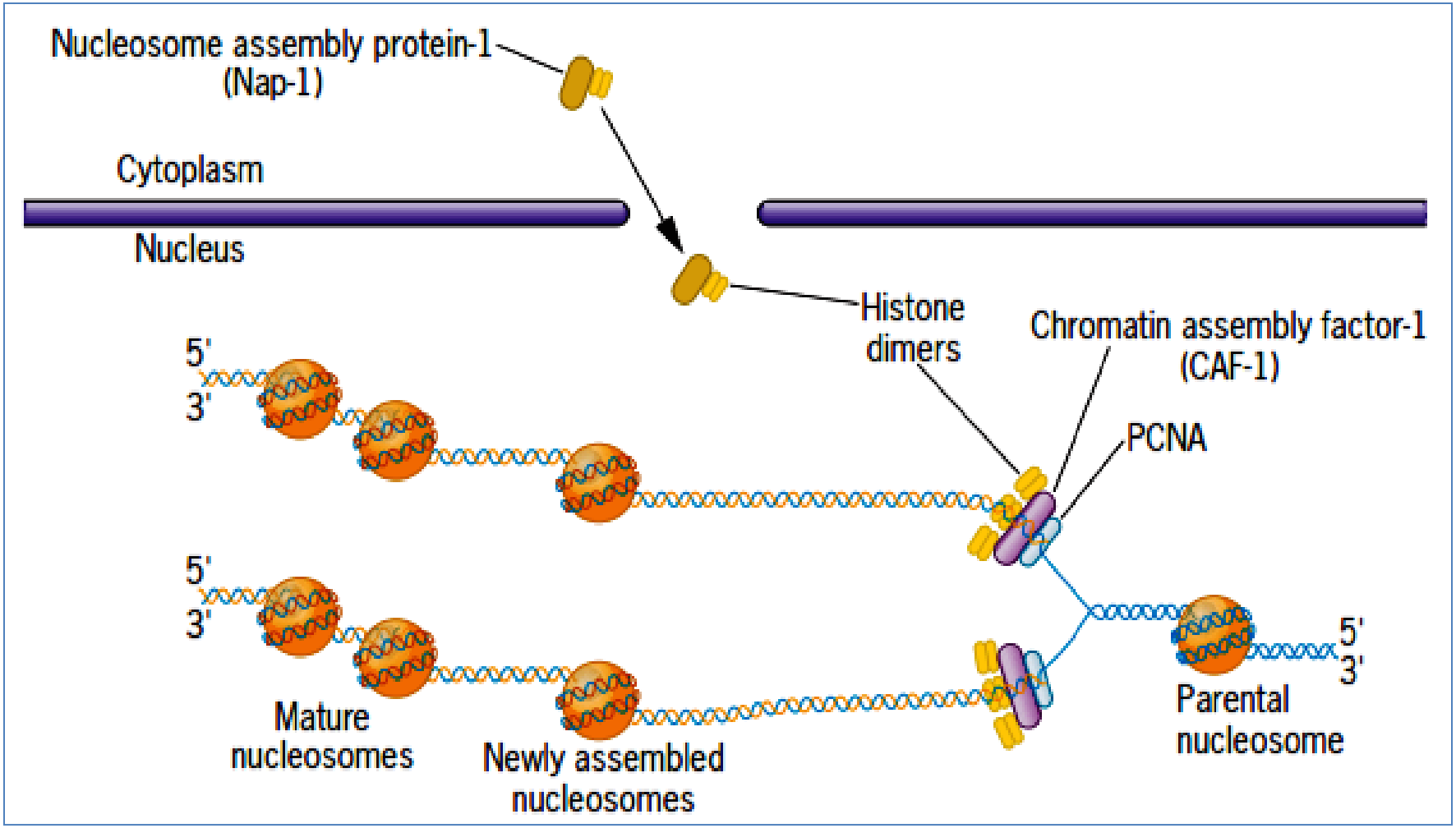
Assembly of the eukaryotic replisome.



Assembly of the eukaryotic replisome.

- As cells enter the S phase, CDK & DDK (2 kinases), are activated.
- DDK phosphorylates loaded Mcm2-7 helicase, and CDK phosphorylates Sld2 and Sld3. Phosphorylated Sld2 and Sld3 bind to Dpb11, and together these proteins facilitate binding of the helicase-activating proteins, Cdc45 and GINS, to the helicase.
- Cdc45 and GINS form a stable complex with the Mcm2-7 helicase (called the Cdc45/ Mcm2-7/GINS, or CMG, complex) and dramatically activate Mcm2-7 helicase activity.
- The leading-strand DNA polymerase (ε) is recruited to the helicase at this stage (before DNA unwinding). After formation of the CMG complex, Sld2, Sld3, and Dpb11 are released from the origin. DNA Pol α/primase and DNA Pol δ (which primarily act on the lagging strand) are only recruited after DNA unwinding.
- The protein–protein interactions that hold the DNA polymerase at the replication fork remain poorly understood. (illustrated as release from Mcm2-7). Exchange of ATP for ADP allows a new round of helicase loading.

Nucleosome assembly during chromosome replication



End Replication Problem

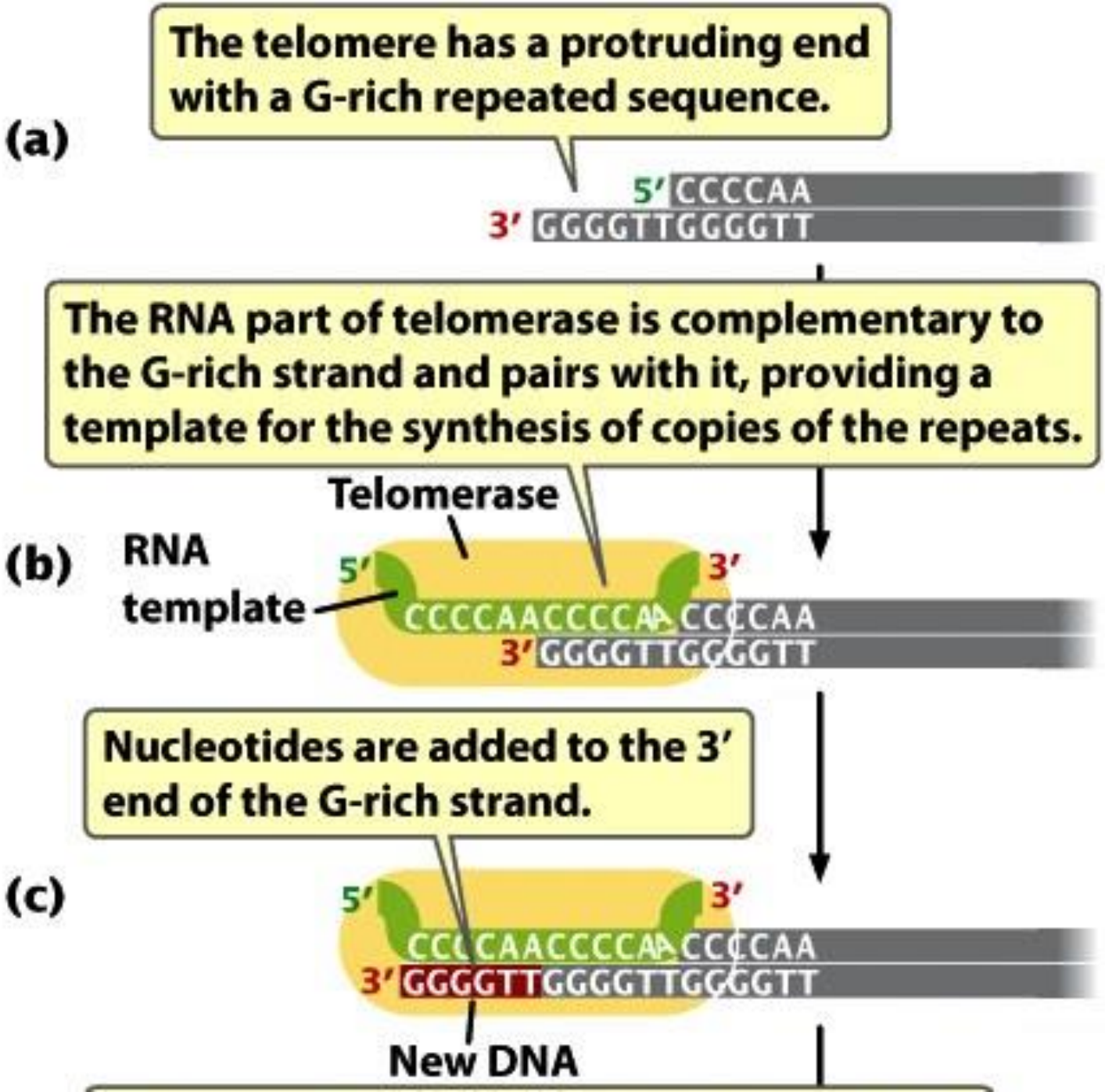
- The RNA primer to initiate replication creates a problem for the replication of the ends of linear chromosomes.
- Once the primer at 5’ end of the chromosome has been removed there remains a 3’ single strand protrusion.
- This gap cannot be filled by DNA polymerases since none of the DNA polymerases can initiate replication. How this gap is to be filled by DNA is called the **end replication problem**.
- The 5’ ends of both the strands face this problem. If the gap is not filled then each round of DNA replication would result in the shortening of one of the two daughter DNA molecules.

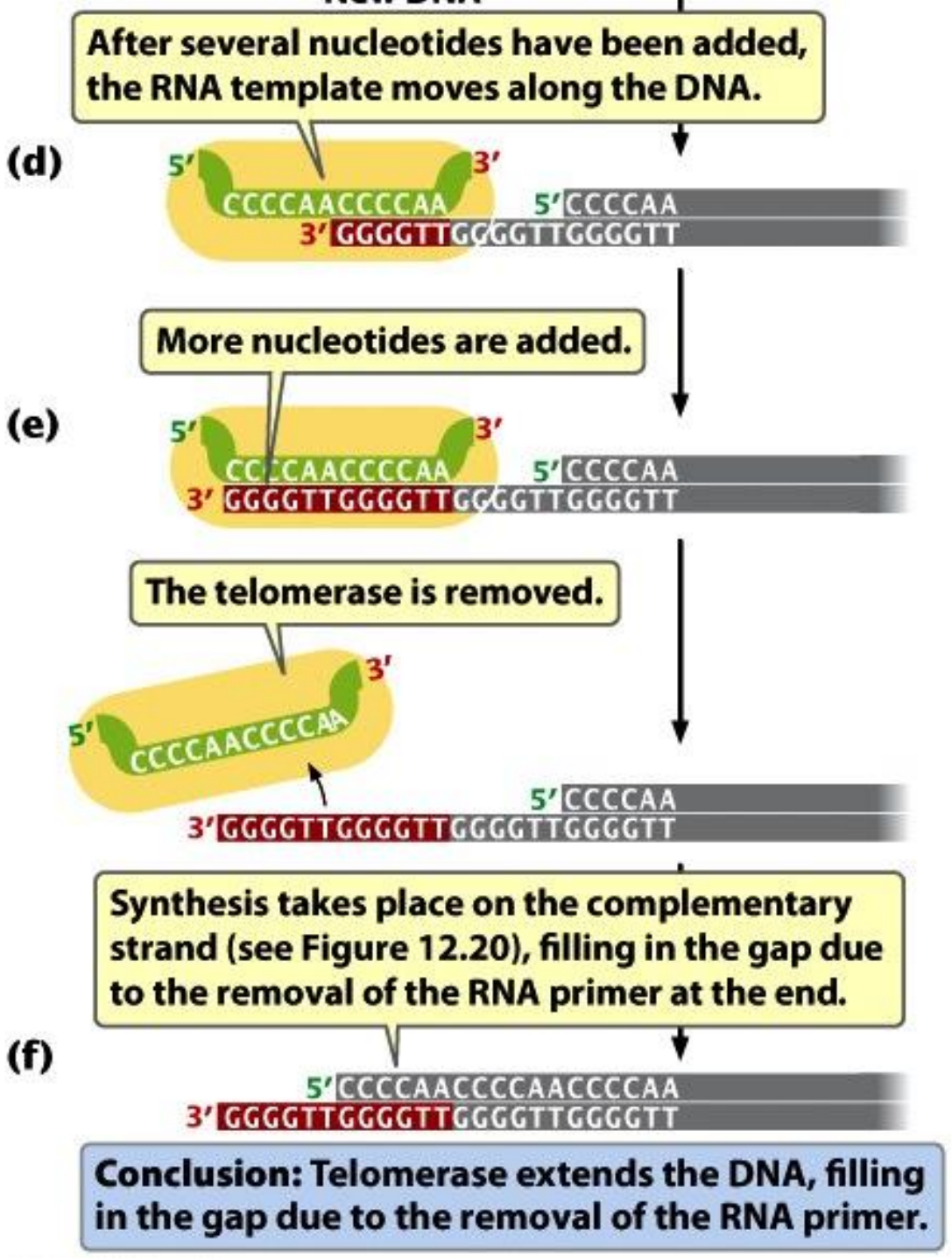
- One solution for **end replication problem** is to use a protein instead of RNA as the primer for the last Okazaki fragment at each end of the chromosome.
- In this situation, the "priming protein" binds to the lagging strand template and uses an amino acid to provide an OH that replaces the 3'OH normally provided by an RNA primer.

- By priming the last lagging strand, the priming protein becomes covalently linked to the 5' end of the chromosome.
- Terminally attached replication proteins of this kind are found at the end of the linear chromosomes of certain species of bacteria (most bacteria have circular chromosomes) and at the ends of the linear chromosomes of certain bacterial and animal viruses.

Telomerase

- Telomerase a RNA dependent DNA polymerase seen in eukaryotes solves the end replication problem as shown in the figure.


The telomere has a protruding end with a G-rich repeated sequence.
(a)
5′ CCCCAA
3′ GGGGTTGGGGTT
The RNA part of telomerase is complementary to the G-rich strand and pairs with it, providing a template for the synthesis of copies of the repeats.
Telomerase
(b)
RNA template
5′
3′
CCCCAACCCCAACCCCAA
3′ GGGGTTGGGGTT
Nucleotides are added to the 3′ end of the G-rich strand.
(c)
5′
3′
CCCCAACCCCAACCCCAA
3′ GGGGTTGGGGTTGGGGTT
New DNA


After several nucleotides have been added, the RNA template moves along the DNA.
(d)
5′
3′
CCCCAACCCCAA
5′CCCCAA
3′GGGGTTGGGGTTGGGGTT
More nucleotides are added.
(e)
5′
3′
CCCCAACCCCAA
5′CCCCAA
3′GGGGTTGGGGTTGGGGTTGGGGTT
The telomerase is removed.
3′
5′
CCCCAACCCCAA
5′CCCCAA
3′GGGGTTGGGGTTGGGGTTGGGGTT
Synthesis takes place on the complementary strand (see Figure 12.20), filling in the gap due to the removal of the RNA primer at the end.
(f)
5′CCCCAACCCCAACCCCAA
3′GGGGTTGGGGTTGGGGTTGGGGTT
Conclusion: Telomerase extends the DNA, filling in the gap due to the removal of the RNA primer.

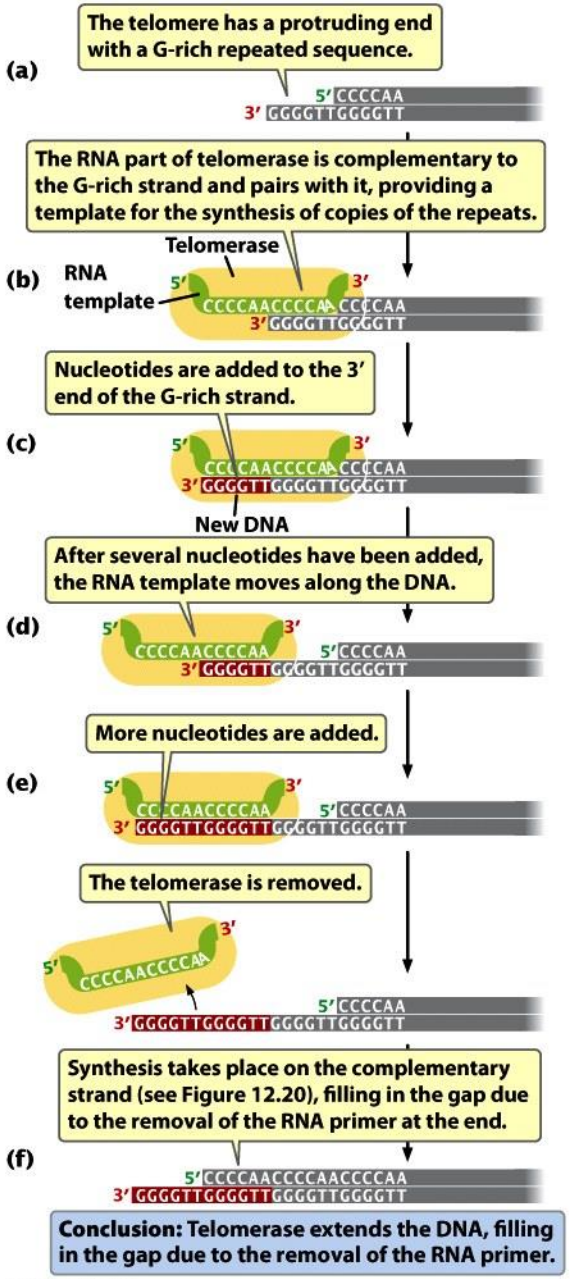
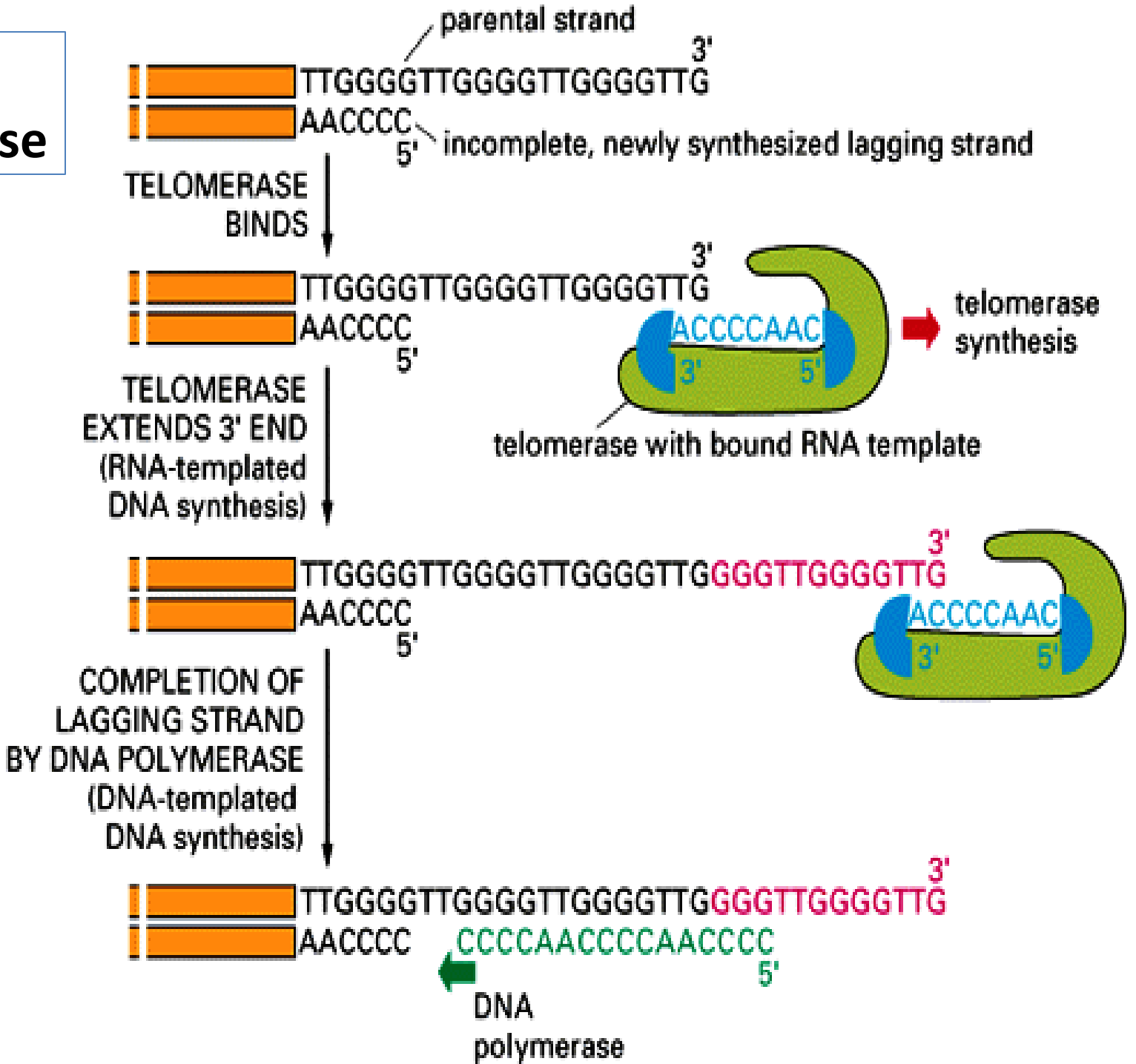


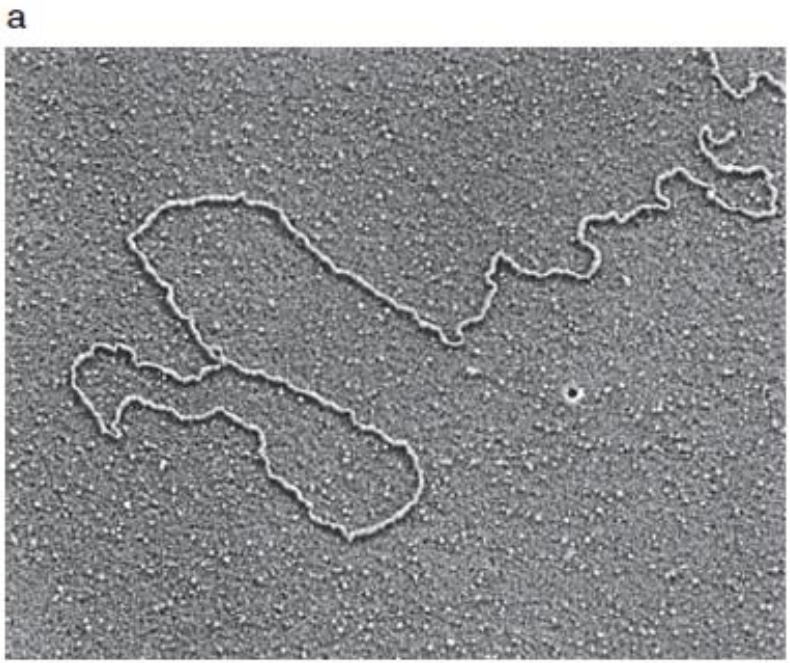
Figure 12-19

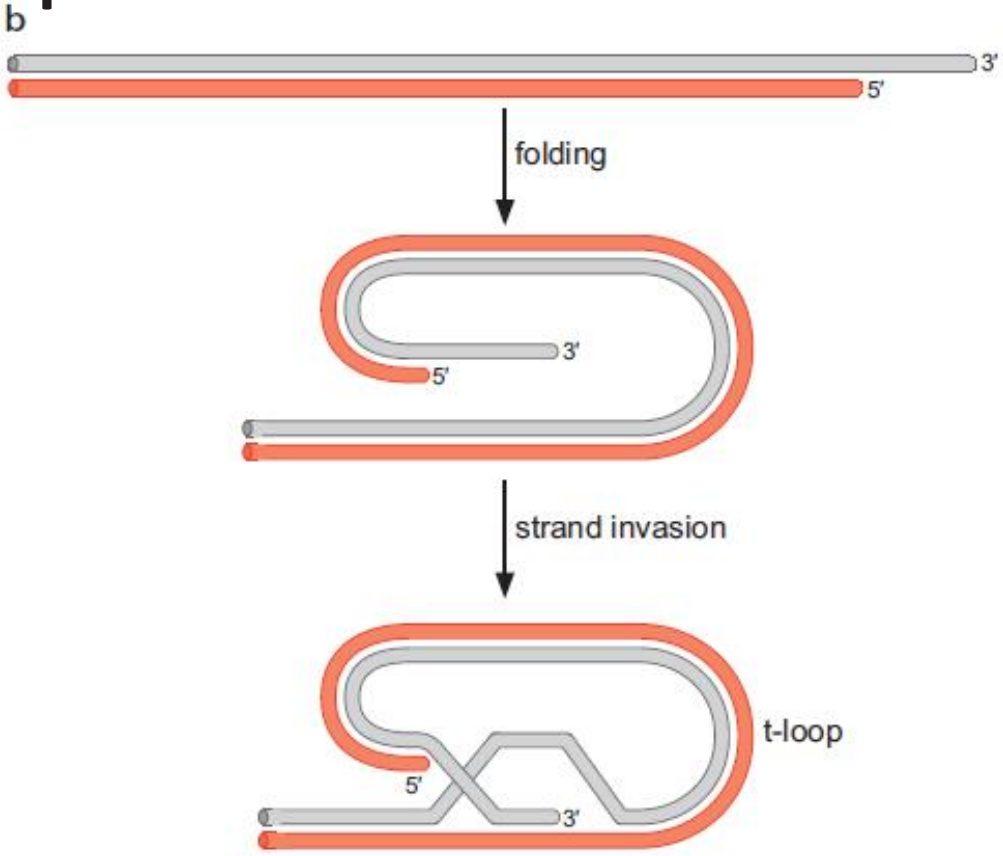
Genetics: A Conceptual Approach, Third Edition

Telomerase


parental strand
3'
TTGGGGTTGGGGTTGGGGTTG
AACCCC
5'
incomplete, newly synthesized lagging strand
TELOMERASE BINDS
3'
TTGGGGTTGGGGTTGGGGTTG
AACCCC
5'
ACCCCAAC
3'
5'
telomerase synthesis
telomerase with bound RNA template
TELOMERASE EXTENDS 3' END (RNA-templated DNA synthesis)
3'
TTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTG
AACCCC
5'
ACCCCAAC
3'
5'
COMPLETION OF LAGGING STRAND BY DNA POLYMERASE (DNA-templated DNA synthesis)
3'
TTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTG
AACCCC
CCCCAACCCCAACCCC
5'
DNA polymerase

Telomeres form a looped structure in the cell.





The loop found at the end of the DNA included the ssDNA at the end of the telomere and is referred to as a t-loop.
The end of the DNA in the upper right-hand corner would be attached to the rest of the chromosome.

THANKS