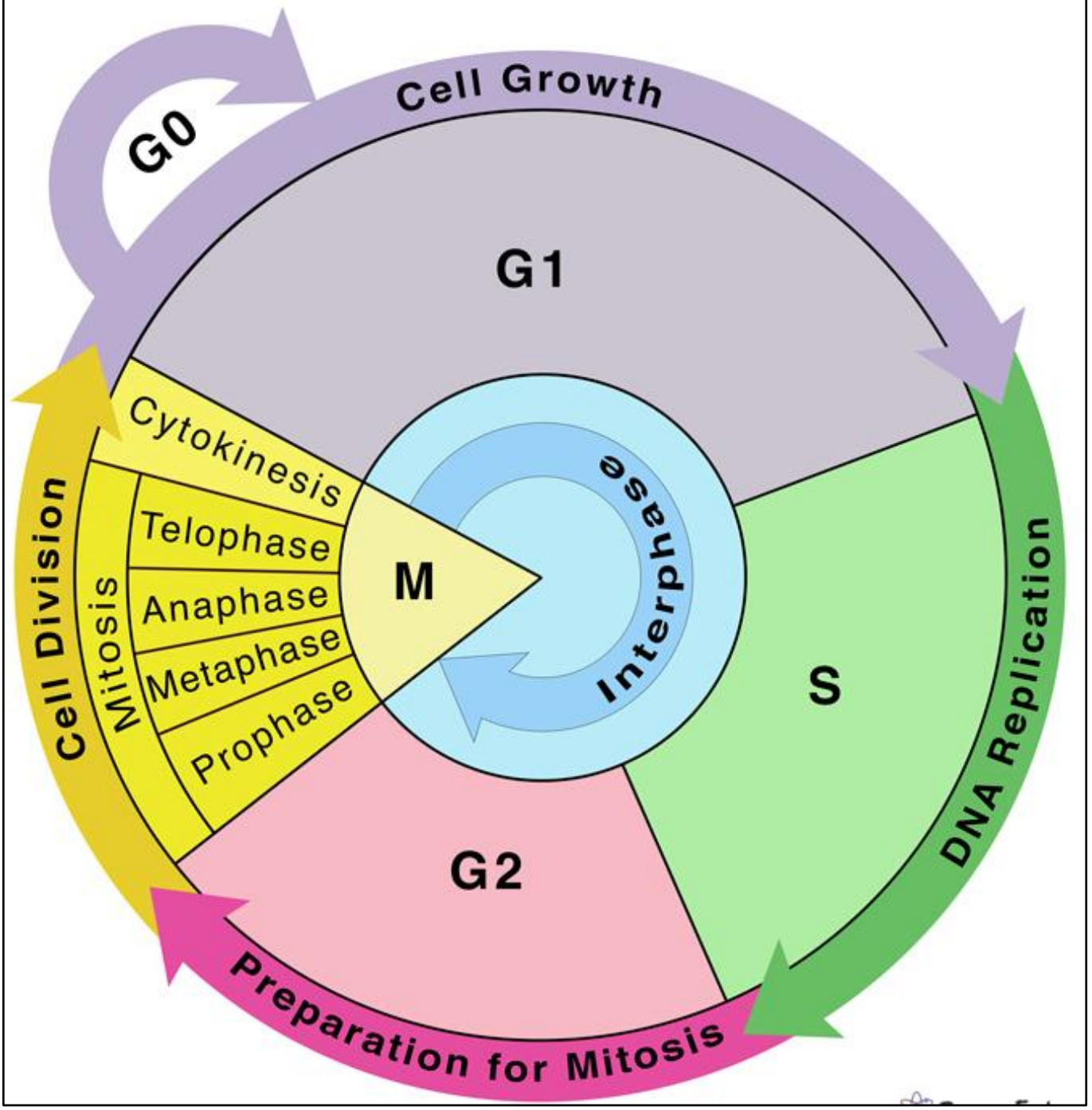
Replication Licensing Factor

To maintain genome integrity in eukaryotes, DNA must be duplicated precisely once before cell division occurs.

A process called replication licensing ensures that chromosomes are replicated only once per cell cycle.

Cell Cycle


G0
Cell Growth
G1
Cytokinesis
Telophase
Anaphase
Metaphase
Prophase
Mitosis
Cell Division
M
Interphase
S
DNA Replication
G2
Preparation for Mitosis

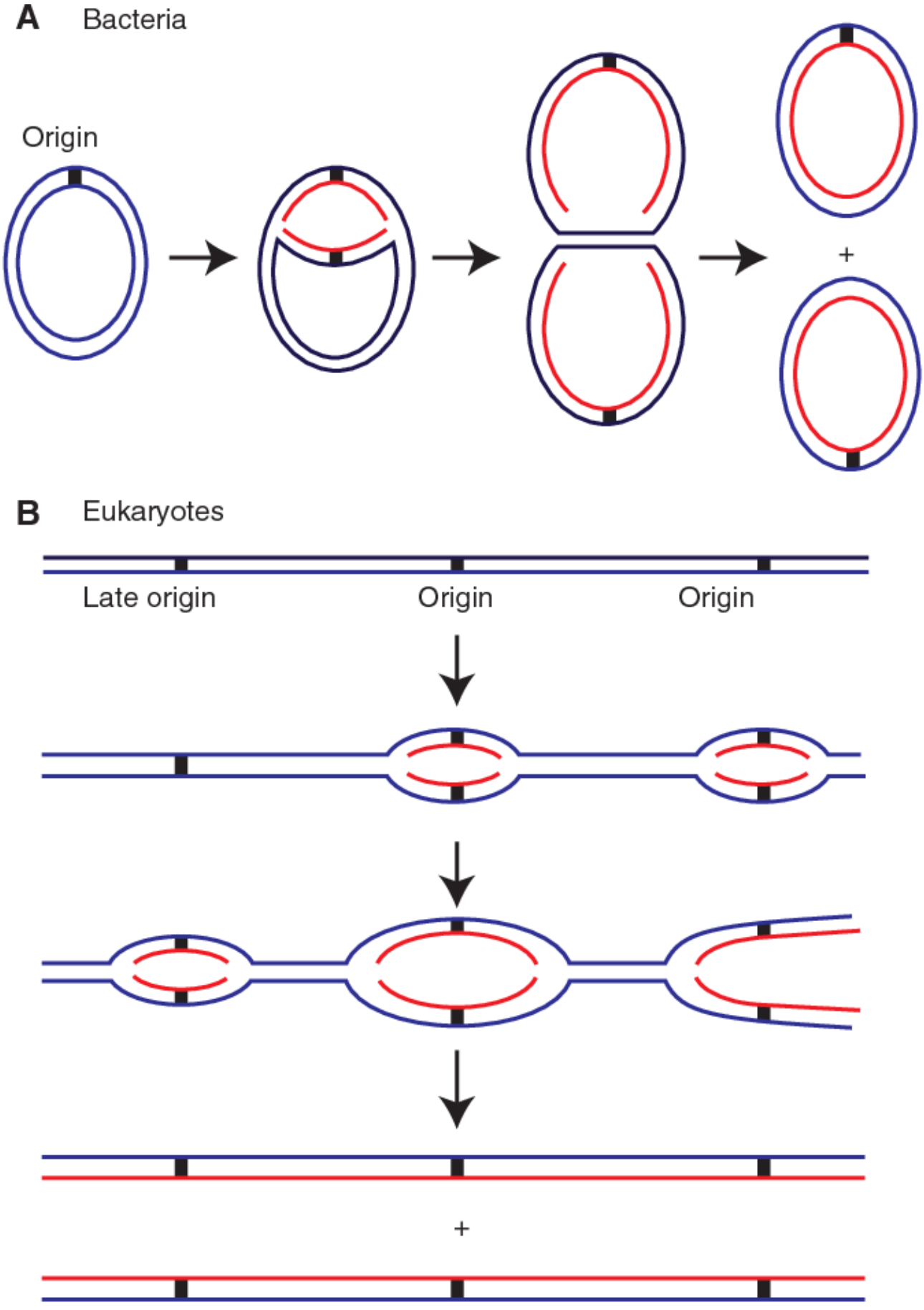
Origin of Replication - Replicon

Prokaryote - Unireplicon

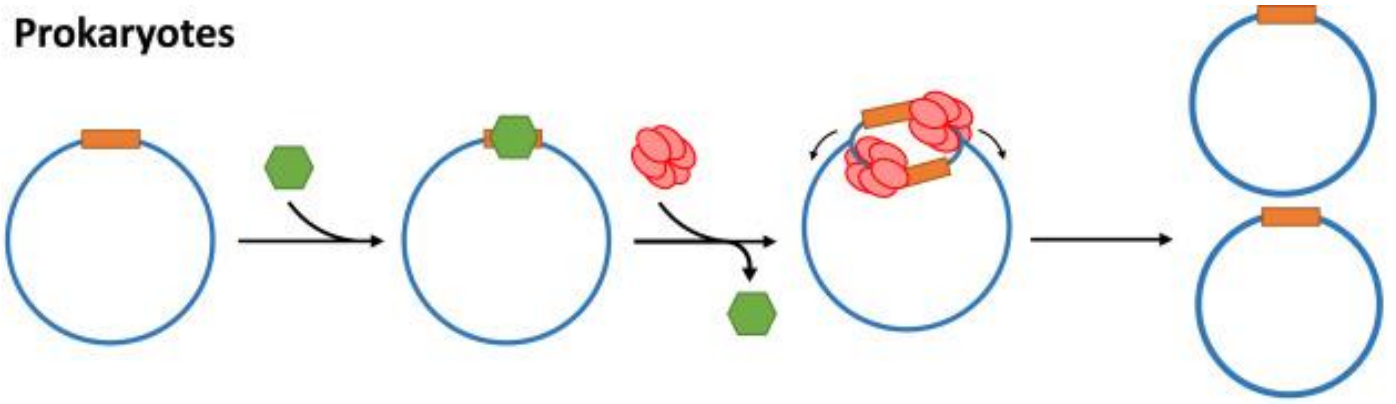
Eukaryote – Multireplicon

Not all activated at once

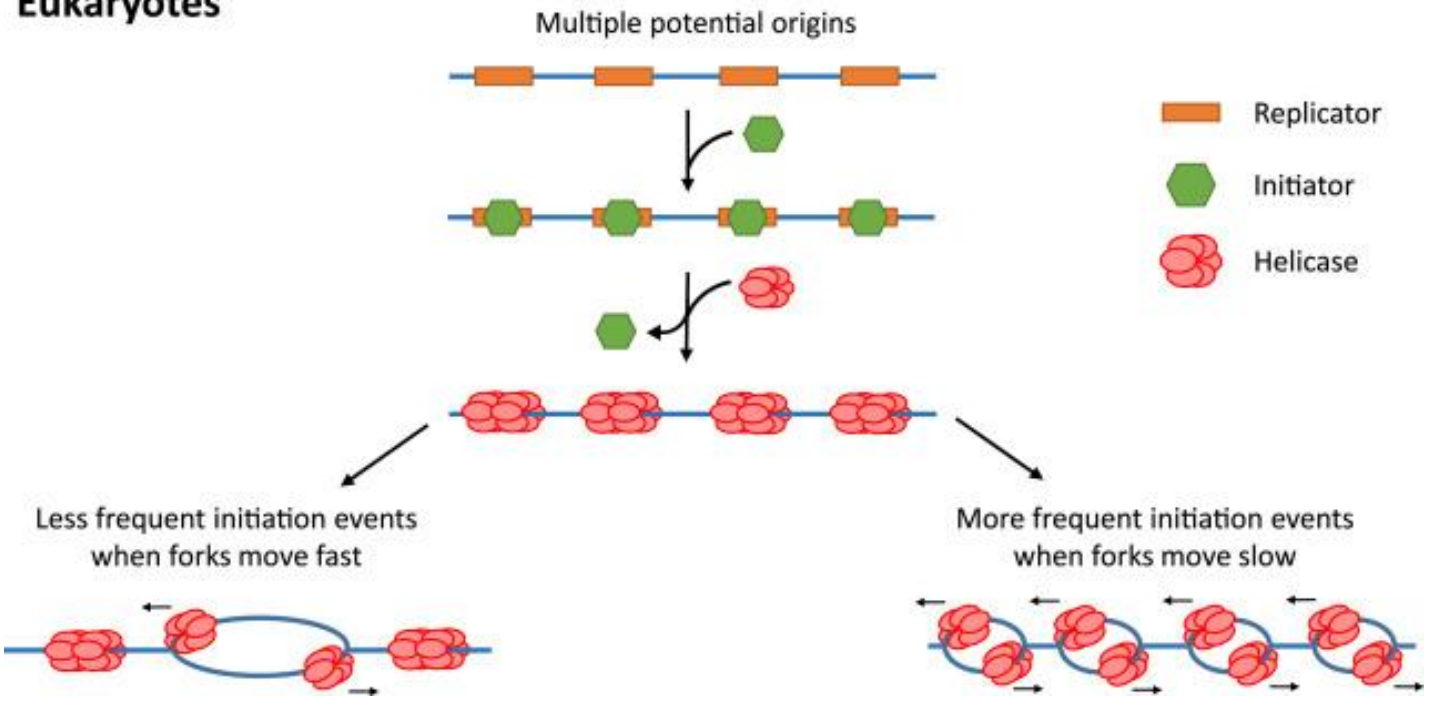
Cluster of 20-28 adjacent are activated sequentially


A
Bacteria
Origin
+
B
Eukaryotes
Late origin
Origin
Origin
+

Prokaryotes



Eukaryotes



A licensing factor is **a protein or complex of proteins that allows an origin of replication to begin DNA replication at that site**. Licensing factors primarily occur in eukaryotic cells, since bacteria use simpler systems to initiate replication.

Origins of replication represent start sites for DNA replication.

Their "firing" must be regulated to maintain the correct karyokinesis of the cell in question.

The origins are required to fire only once per cell cycle, controlled by **replication licensing factors.**

If the origins were not carefully regulated then DNA replication could be restarted at that origin giving rise to multiple copies of a section of DNA.

This could be damaging to cells and could have detrimental effects on the organism as a whole.

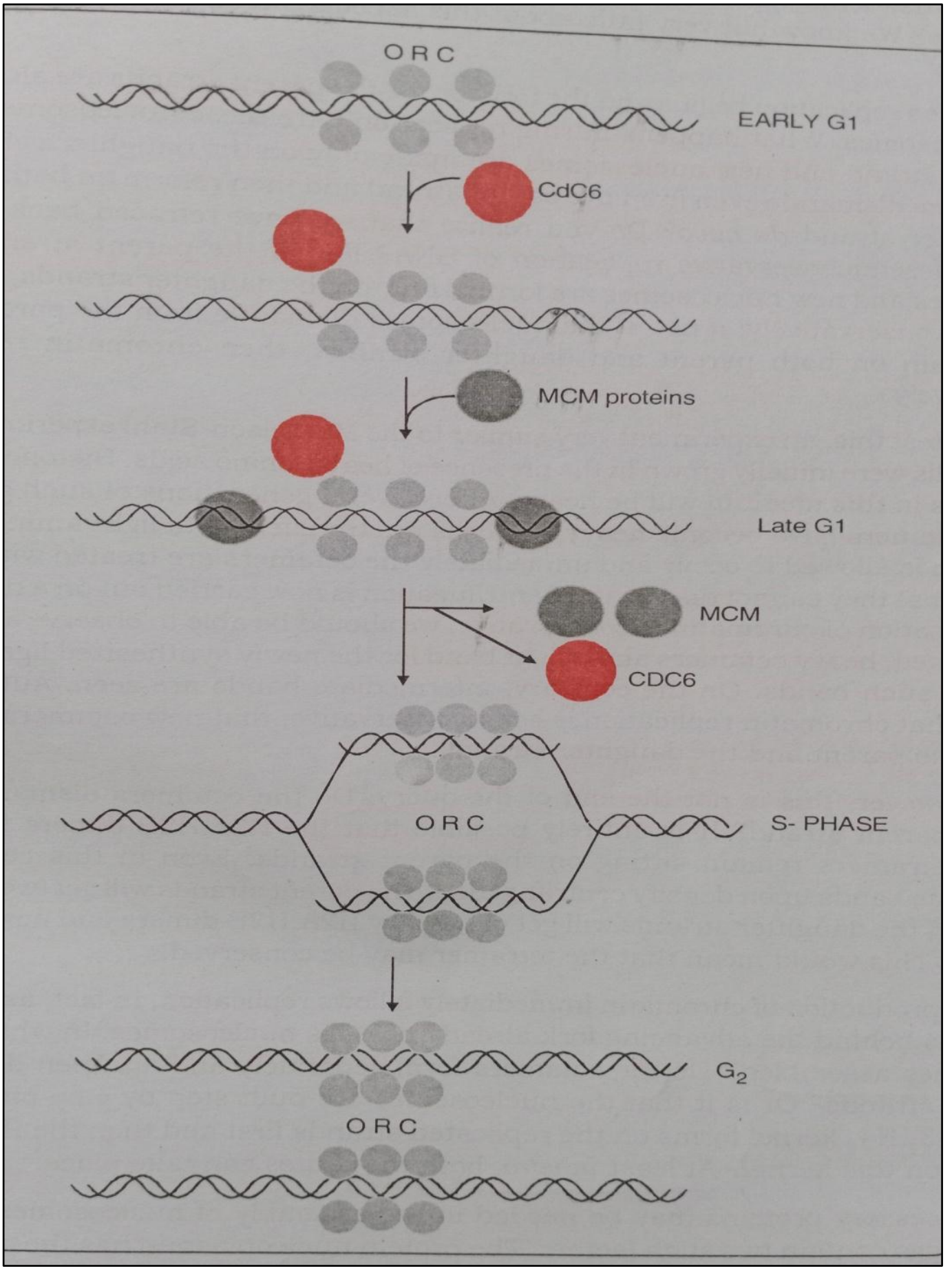
During G1 phase – All Autonomous Replicating Sequences (ARSs) are bound by a group of proteins, forming Origin Recognition Complex (ORC)

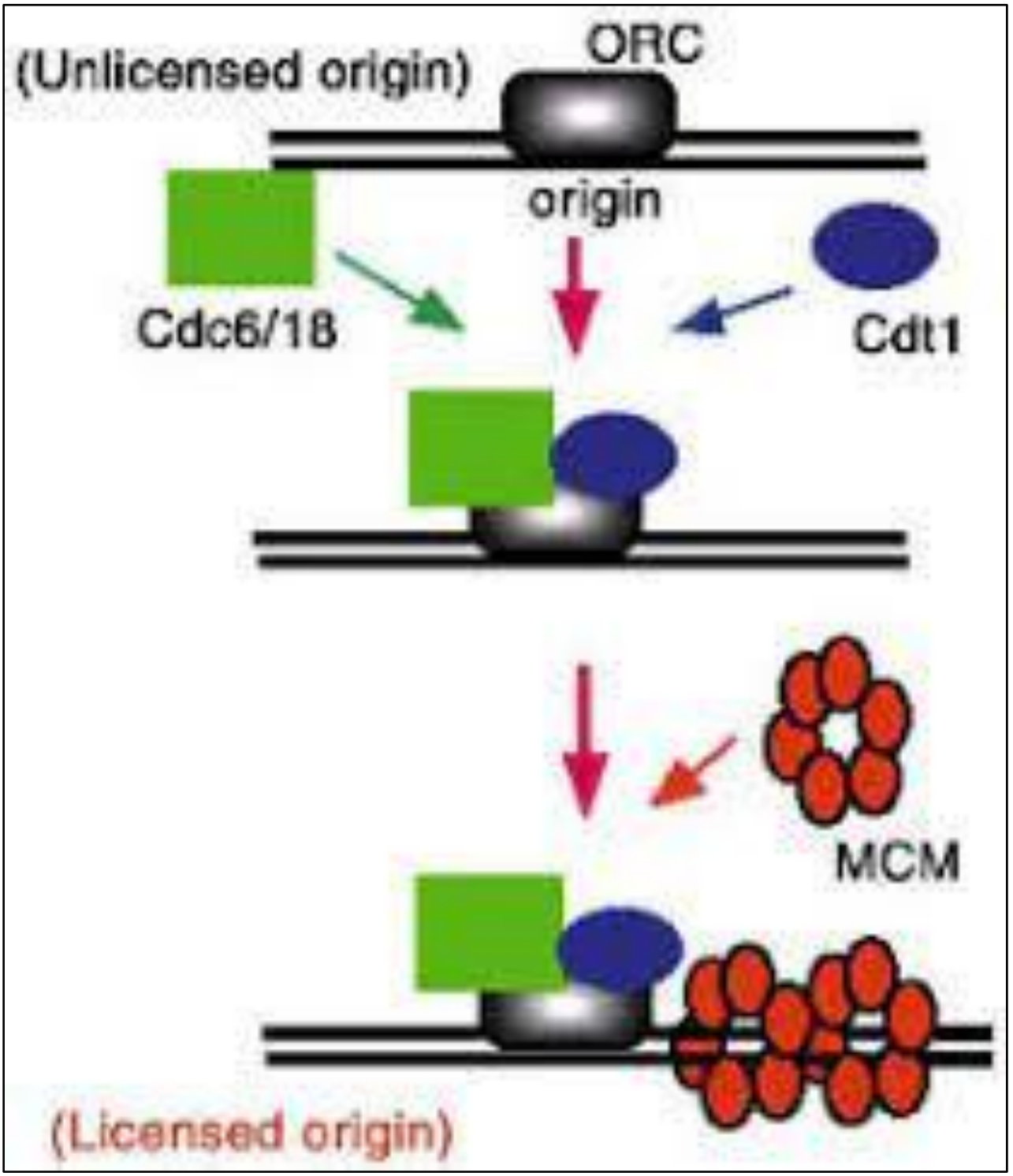
These recognition complexes are found in G1, but synthesis is not initiated at their sites until S phase.
There must be still other proteins involved in the actual initiation signal. Most important of these proteins are specific kinases, key enzyme involved in phosphorylation, that are integral part of cell cycle control.

Bound along with ORC, a prereplication complex is formed that is accessible to DNA polymerase.

After these kinases are activated, they serve to complete the initiation complex, directing localized unwinding by the helicase enzyme and triggering DNA synthesis.

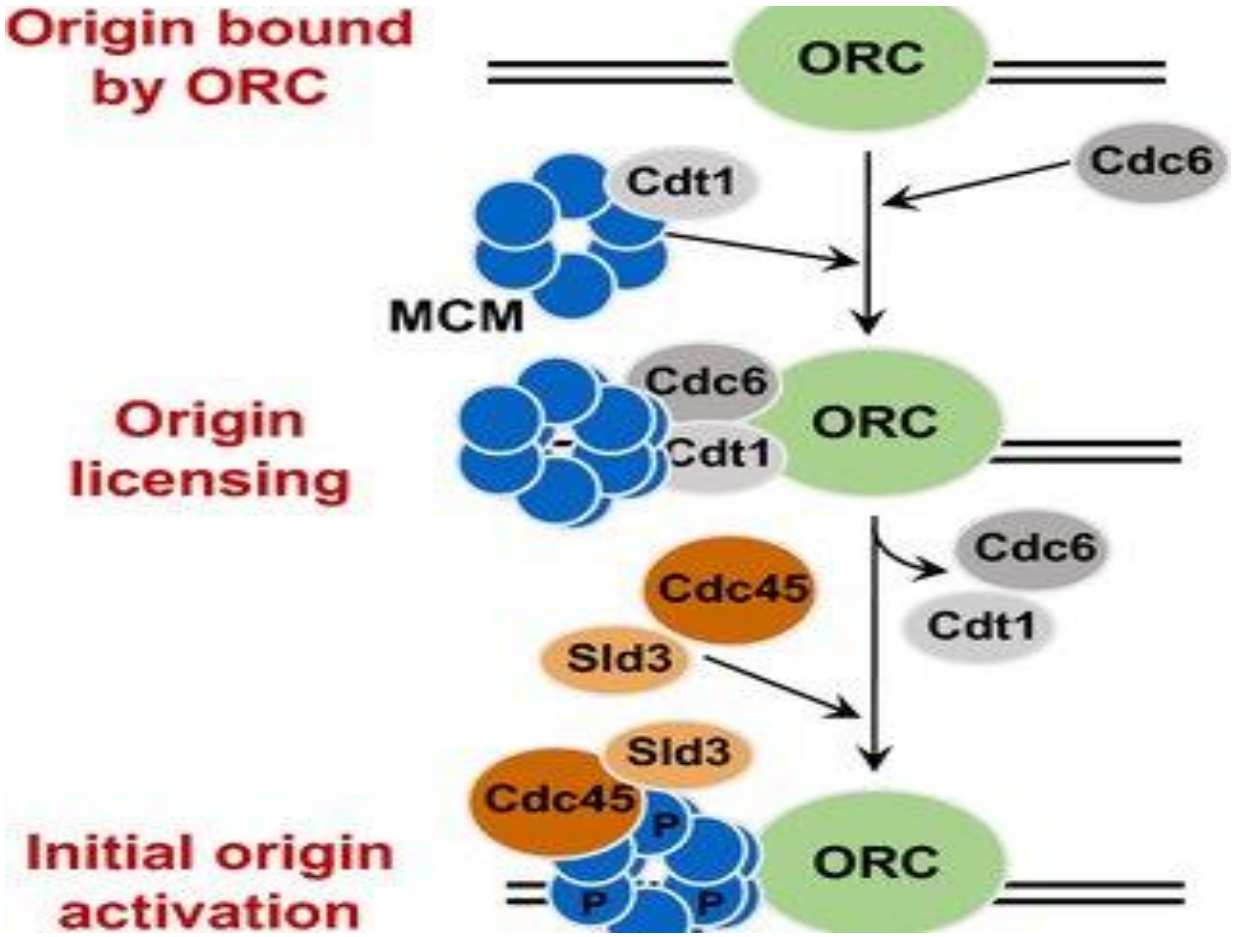
Activation also inhibits reformation of the prereplication complexes once DNA synthesis has been completed at each replicon. It ensures that replication occurs only once along each stretch of DNA during each cell cycle.


ORC
EARLY G1
CdC6
MCM proteins
Late G1
MCM
CDC6
ORC
S- PHASE
G2
ORC


(Unlicensed origin)
ORC
origin
Cdc6/18
Cdt1
MCM
(Licensed origin)

- Immediately after mitosis has finished for once, the cell again enter G1 phase for the next round of cell cycle.
- Cdc6 and Cdt1 proteins are synthesised in G1 phase.
- These two together bind to the origin recognition complex (ORC), which is already bound at the origin and in fact never leaves these sites throughout the cycle.

Now we have a so-called pre-replication complex, which then allows a heterohexameric protein complex of proteins MCM2 to MCM7 to bind. This entire hexamer exerts its activity untill helicase unwind the double stranded DNA.



At this point Cdc6 leaves the complex and is inactivated, by being degraded in yeast but by being exported from the nucleus in metazoans, triggered by CDK-dependent phosphorylation.

The next steps included the loading of a variety of other proteins like MCM10, a CDK, DDK and Cdc45, the latter directly required for loading the DNA polymerase. During this period Cdt1 is released from the complex and the cell leaves G1 phase and enters S phase when replication starts.

Cdc6 and Cdt1 fulfil the role of licensing factors. They are only produced in G1 phase, in addition to which binding of all the proteins in this process excludes binding of additional copies. In this way their mode of action is limited to starting replication once, since once they have been ejected from the complex by other proteins, the cell enters S phase, during which they are not re-produced or re-activated. Thus they act as licensing factors, but only together they can act.

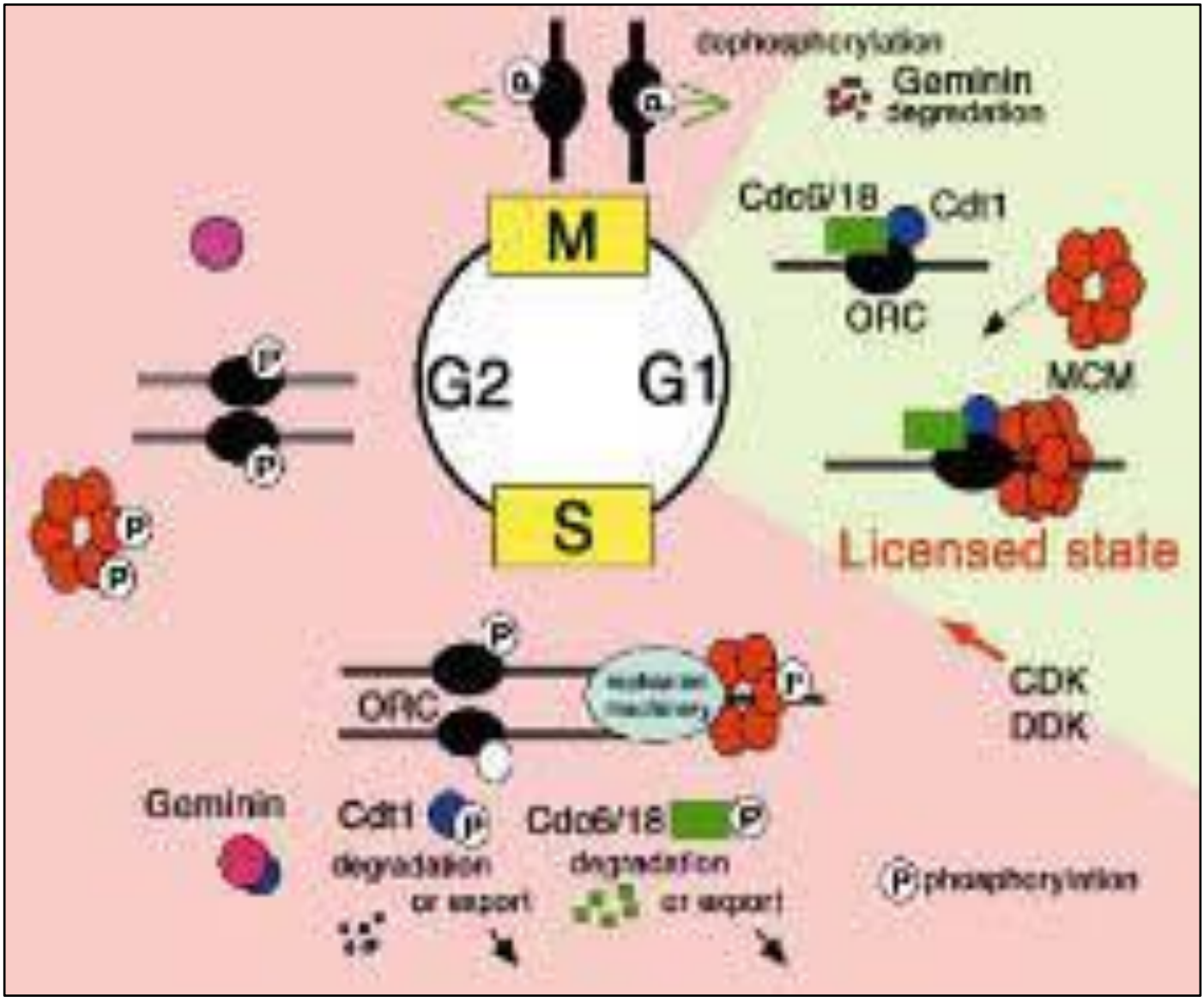
Its control has been uncovered by the discovery of the CDKs (cyclin dependent kinases) as master regulators of the cell cycle and the initiator proteins of DNA replication, such as the Origin Recognition Complex (ORC), Cdc6/18, Cdt1 and the MCM complex.

At the end of mitosis, the MCM complex is loaded on to chromatin with the aid of ORC, Cdc6/18 and Cdt1, and chromatin becomes licensed for replication. CDKs, together with the Cdc7 kinase, trigger the initiation of replication, recruiting the DNA replicating enzymes on sites of replication.

The activated MCM complex appears to play a key role in the DNA unwinding step, acting as a replicating helicase and moves along with the replication fork, at the same time bringing the origins to the unlicensed state. The cycling of CDK activity in the cell cycle separates the two states of replication origins, the licensed state in G1-phase and the unlicensed state for the rest of the cell cycle. Only when CDK drops at the completion of mitosis, is the restriction on licensing relieved and a new round of replication is allowed.

Such a CDK-regulated licensing control is conserved from yeast to higher eukaryotes, and ensures that DNA replication takes place only once in a cycle. *Xenopus laevis* and mammalian cells have an additional system to control licensing.

Geminin, whose degradation at the end of mitosis is essential for a new round of licensing, has been shown to bind Cdt1 and negatively regulate it, providing a new insight into the regulation of DNA replication in higher eukaryotes.


dephosphorylation
Geminin
degradation
M
G2
G1
S
Cdc6/18
Cdt1
ORC
MCM
Licensed state
CDK
DDK
ORC
Geminin
Cdt1
degradation
or export
Cdc6/18
degradation
or export
P phosphorylation

Helicase loading and its activation are regulated -by the level of the protein Cyclin Dependent Kinase (CDK)

During G_1 the CDK level is low

Allowing helicase loading but preventing helicase activation.

Entry into S phase is coupled with increase in CDK level and activation of loaded helicases by CDK
But simultaneously preventing new helicase loading.

The high level of CDK during S, G_2 and M phases inhibits the function of ORC and cdc6 and cdt1 (the two helicase loading proteins). It is only when cells complete cell division that CDK activity is eliminated, allowing a new round of helicase loading to commence.

Regulatory mechanisms of the cell to ensure that each replicon replicates only once in one cell cycle of cell division, mediated by a factor known as Replication Licensing Factor, which permit the formation of initiation complex.

The licensing factors present in the cytoplasm of the cell

This factor must get into the nucleus where DNA is scheduled to replicate

So the factor can get entry into the nucleus only when the nuclear membrane is dissolved

After one round of mitosis, the factor will be within the nucleus

After replication the factor is destroyed

Thus only one round of replication can takes place by the captured factor.

If second round of replication is to happen, the factor has to get entry again afresh, which is possible only after another mitosis.

ORC is always bound to DNA except in the elongation phase.
This quality of ORC argues against its being the initiating/licensing factor.

In the yeast, the prime candidates for licensing factor(s) are the MCM proteins – MCM2, 3, 5.

These proteins are required for replication and enter the nucleus only during mitosis.

In animal cells though, the MCM proteins may just be one of the components of the licensing factor.

This is so because the MCM proteins are inside the nucleus throughout the cell cycle.

It may therefore be that some other protein may be required to assist MCM protein in initiating replication.

Cell cycle protein Cdc6 in replication licensing

1. It is highly unstable protein with a half-life less than 5 minutes, thus degrade rapidly which will not allow it to support replication initiation more than once.

2. Its synthesis in G_1 phase and binding with ORC between mitosis and G_1 phase also fits that this is the licensing factor.

Already bound ORC to the origin probably guides Cdc6 at the origin.

Once Cdc6 binds, it helps MCM proteins to bind to the complex.

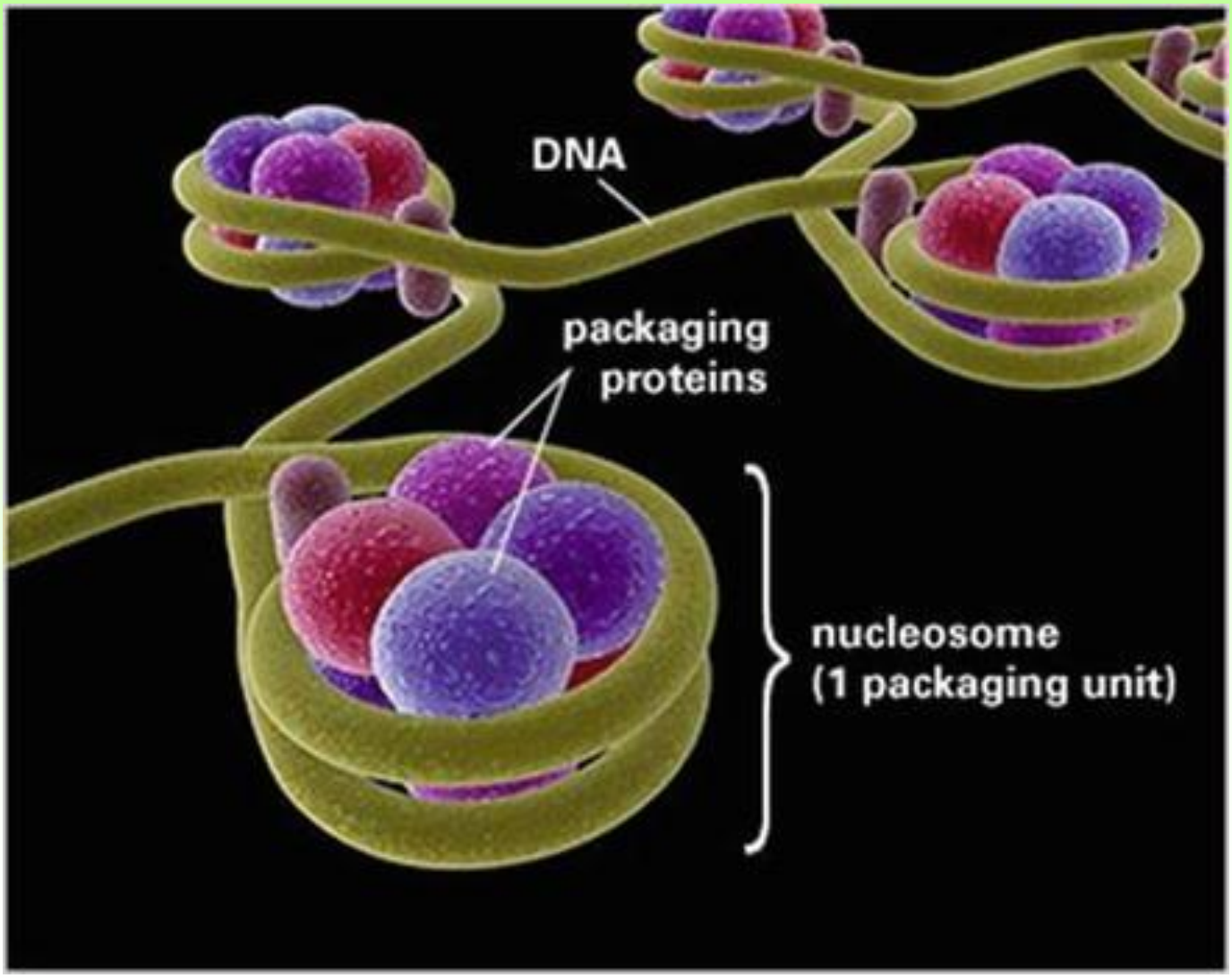
Prereplication complex is made up of ORC, Cdc6 and MCM proteins - in the beginning of the S phase.

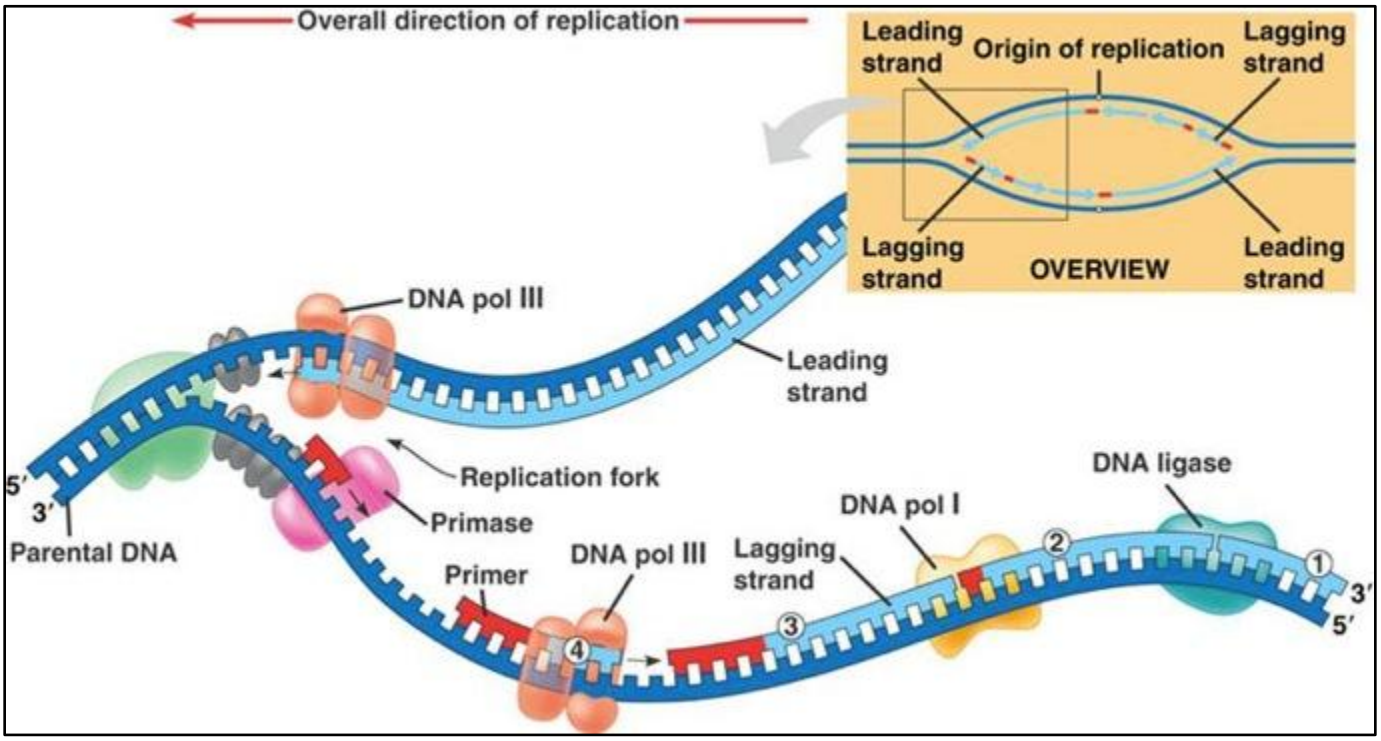
Immediately upon initiation, Cdc6 and MCM are displaced from the origin leaving just bound ORC.

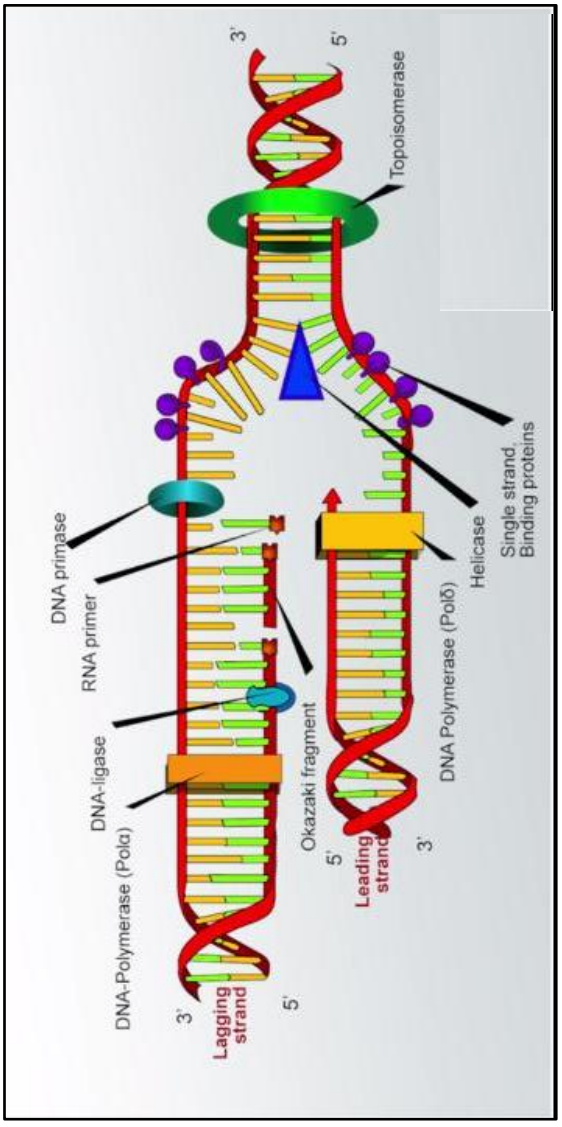
Cdc6 is rapidly degraded, probably through ubiquitination, thus not available throughout the S phase.

Without Cdc6, MCM proteins cannot bind again and thus a second initiation becomes impossible unless the cell undergoes mitosis paving way for entry of fresh Cdc6.

ASSEMBLY OF NEW NUCLEOSOME




Overall direction of replication
Leading strand
Origin of replication
Lagging strand
Lagging strand
OVERVIEW
Leading strand
DNA pol III
Leading strand
5′
3′
Parental DNA
Replication fork
Primase
Primer
DNA pol III
Lagging strand
DNA pol I
DNA ligase
1
2
3
4
3′
5′


3'
5'
Topoisomerase
DNA primase
RNA primer
DNA-ligase
DNA-Polymerase (Polα)
Okazaki fragment
Leading strand
DNA Polymerase (Polδ)
Helicase
Single strand Binding proteins
3'
5'
Lagging strand
5'
3'

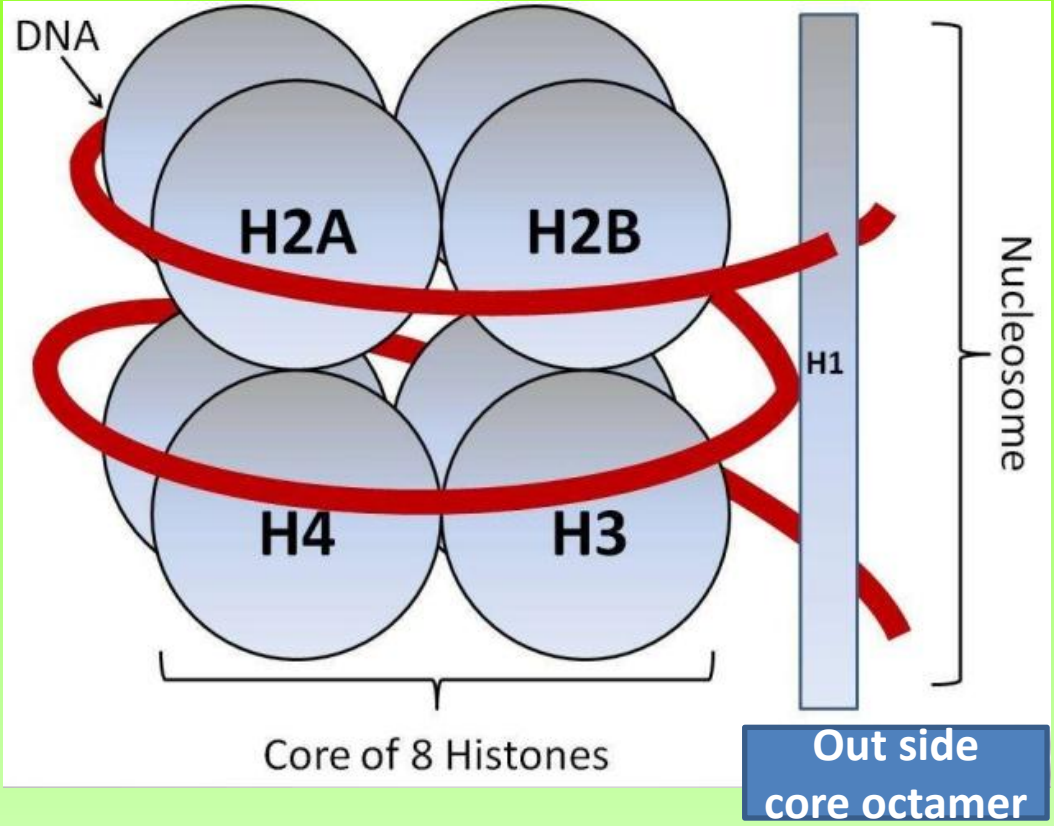
Replication

Replication of chromatin

Replication of DNA and reconstruction of chromatin

Addition of histones at the growing fork

Very little known



Nucleosome disassembly

DNA is rapidly repackaged into nucleosomes in an ordered series of events

First step is the binding of an H3.H4 tetramer

Two H2A.H2B dimers associate later

H1 joins this complex last, presumably during formation of higher order chromatin assemblies

Half of the nucleosomes newly synthesized

- If old histones lost completely, it would erase all “memory” of the previously modified nucleosomes

- If old histones retained on a single chromosome, that chromosome would have a distinct set of modifications relative to the other chromosome.

Old histones are present on both daughter chromosomes

Mixing is not entirely random

H3.H4 tetramers in one nucleosome composed of either all new or all old histones

As the replication fork passes, nucleosomes are broken down into their component subassemblies

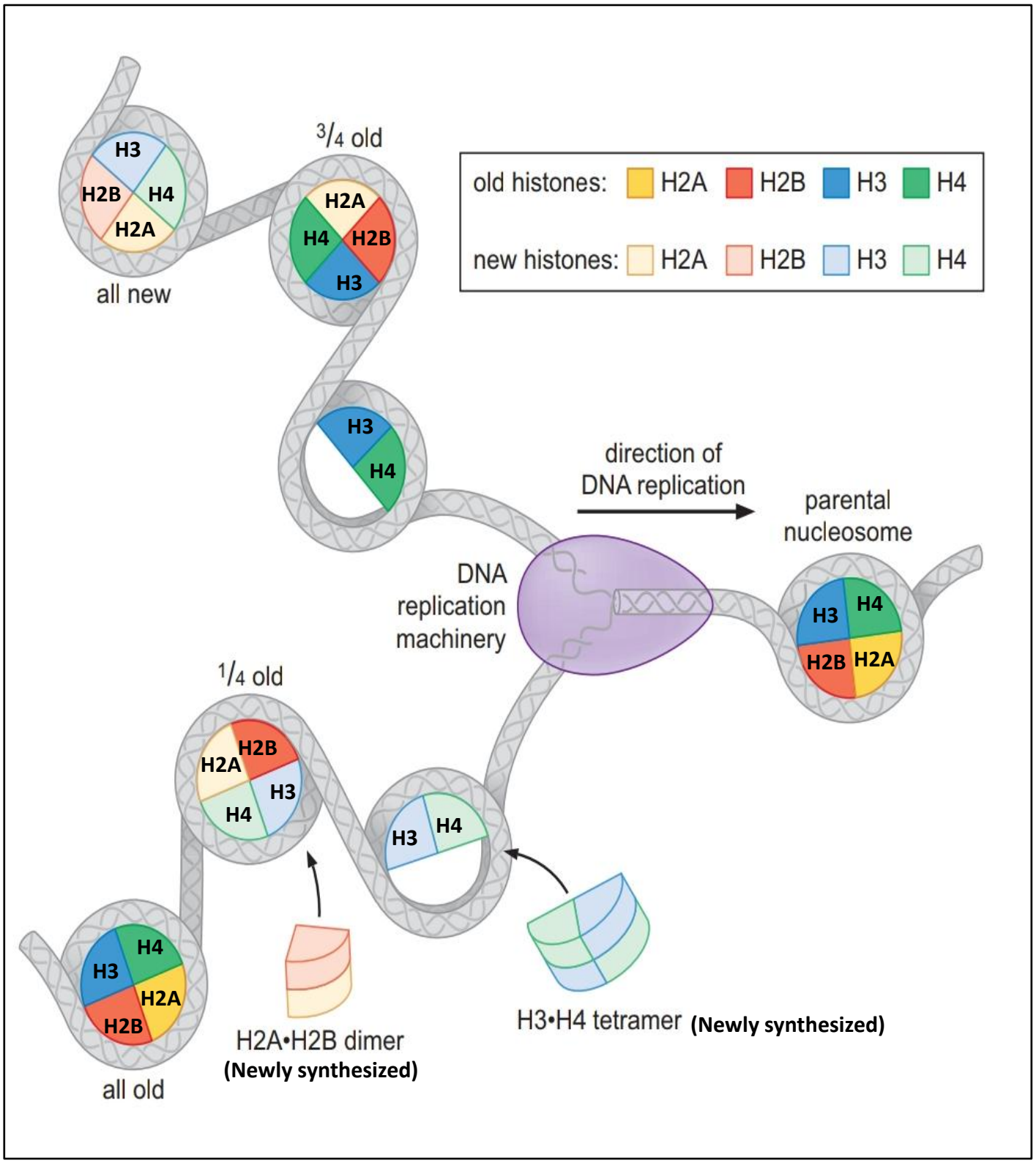
H3.H4 tetramers appear to remain bound to one of the two daughter duplexes at random and are never released from DNA into the free pool of histones

In contrast, the H2A.H2B dimers are released and enter the local pool, available for new nucleosome assembly

- **Old modified histones will tend to rebind one of the daughter chromosomes at a position near their previous position on the parental chromosome**

- **Old histones have an equal probability of binding either daughter chromosome**

- **This localized inheritance of modified histones ensures that a subset of the modified histones is localized in similar positions on each daughter chromosome.**


H3
H2B
H4
H2A
all new
3/4 old
H2A
H4
H2B
H3
old histones: H2A H2B H3 H4
new histones: H2A H2B H3 H4
H3
H4
direction of
DNA replication
parental
nucleosome
DNA
replication
machinery
H3
H4
H2B
H2A
1/4 old
H2B
H2A
H3
H4
H3
H4
H4
H3
H2A
H2B
all old
H2A•H2B dimer
(Newly synthesized)
H3•H4 tetramer (Newly synthesized)

Assembly is not spontaneous

Factors required to direct the assembly

Extracts from *Xenopus* eggs contain two such factors

Nucleoplasmin has the ability of binding H2A and H2B while the protein N1 binds H3 and H4

Job of these accessory proteins seems to hold the different components of octamers and escort them to sites of nucleosome assembly and to release them in a controlled manner leading to the correct assembly of the nucleosomes

These factors have been referred to as histone chaperones.

Acetylation of histones H3 and H4 seems to be an event necessary for chromatin replication

Shortly after their synthesis, histones H3 and H4 associate with each other and are acetylated at a number of lysine residues within their amino-terminal domain

In higher eukaryotes, this acetylation is transient and residues are rapidly deacetylated after the histones have been packaged into chromatin.

The acetylation of histone prior to their packaging into chromatin is carried out by enzyme known as B-type histone acetyltransferases (B-type HATs). Such enzymes are different from other (A-type HATs) acetyltransferases which acetylate chromosomal histones.

The only known and characterised B-type HAT is Hat-1.

This enzyme is widely conserved and at least in *Xenopus* and humans can acetylate lysine residues 5 and 12 of histone H4.

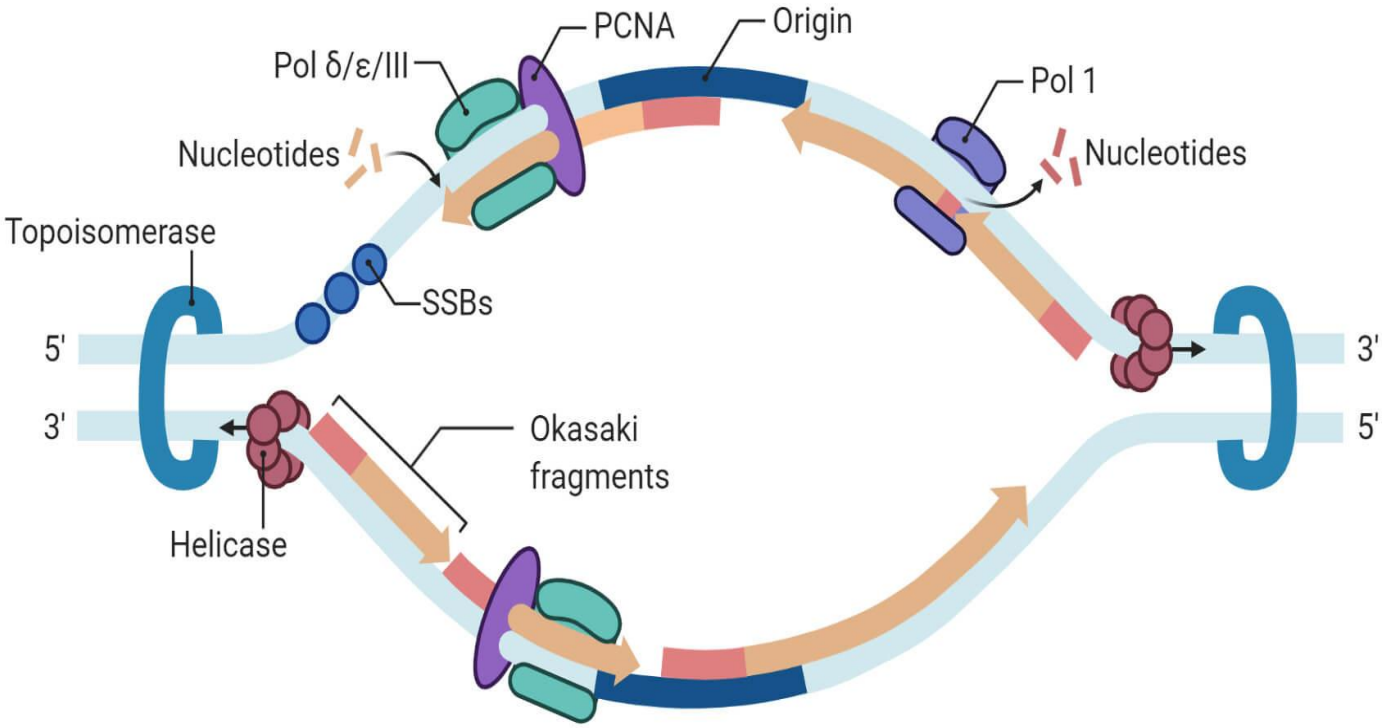
There may be other enzymes in the cells which can carry out the same acetylation.

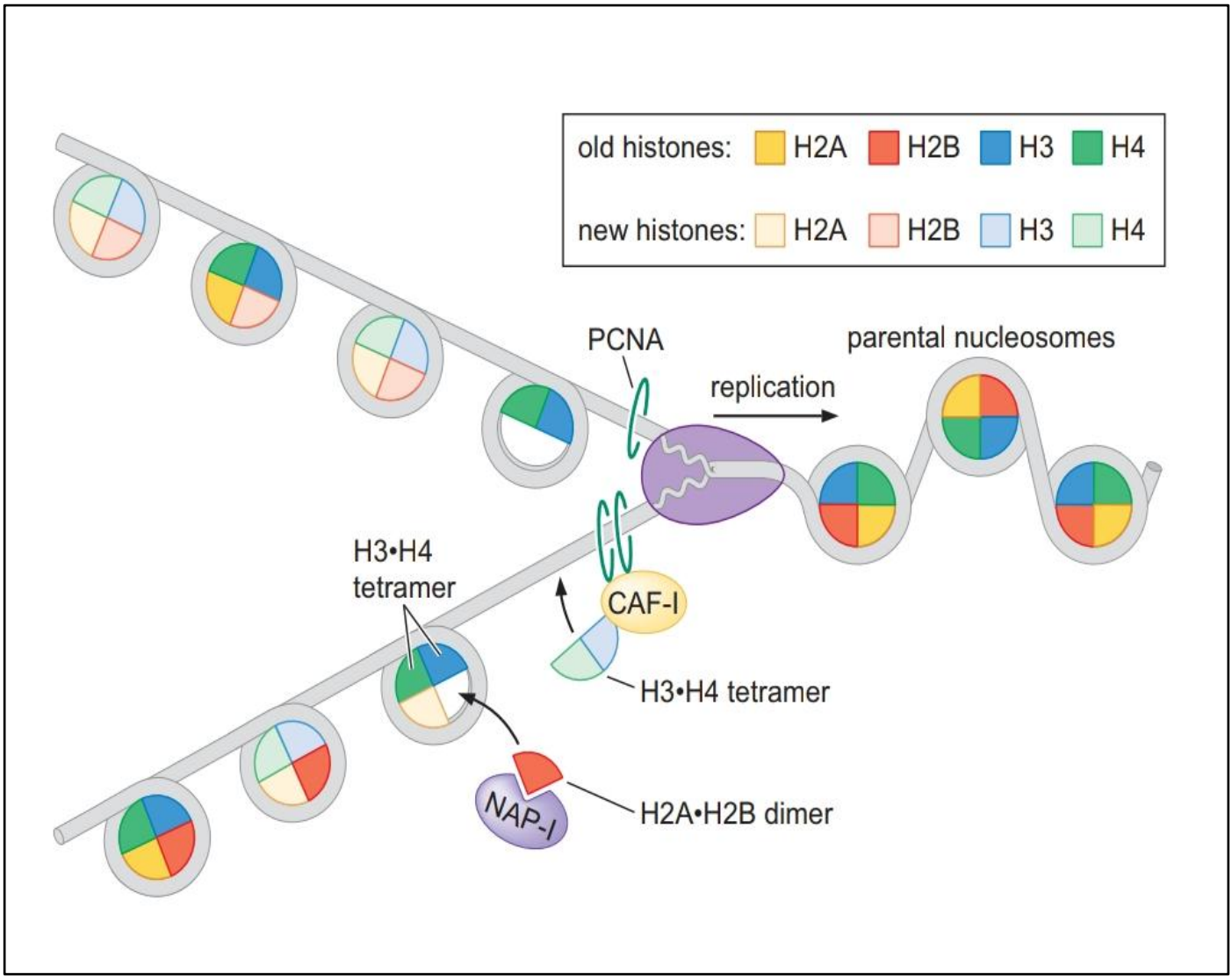
Acetylation of lysine 8 of H4 also occur, which is sufficient for viability of nucleosome assembly. But this acetylation must be carried out by a different B-type HAT.

We know nothing about the B-type HATs which acetylate H3, except that they must exist.

However, unlike H4 where acetylation of lysine 5 and 12 was a conserved feature across many species, the H3 acetylation points differ from species to species.

DNA ELONGATION COMPLEX





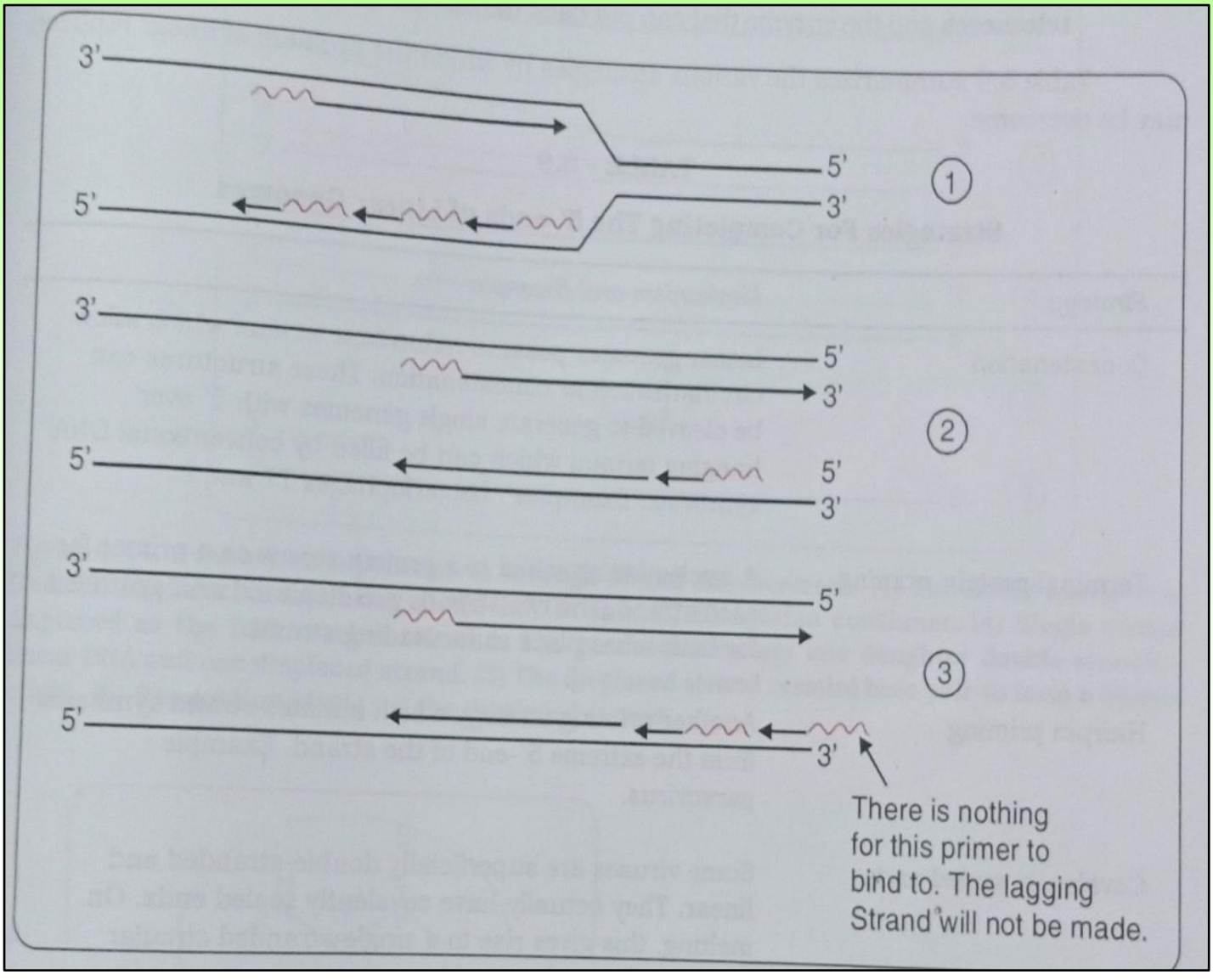
Chromatin Assembly Factor 1 (CAF-1)

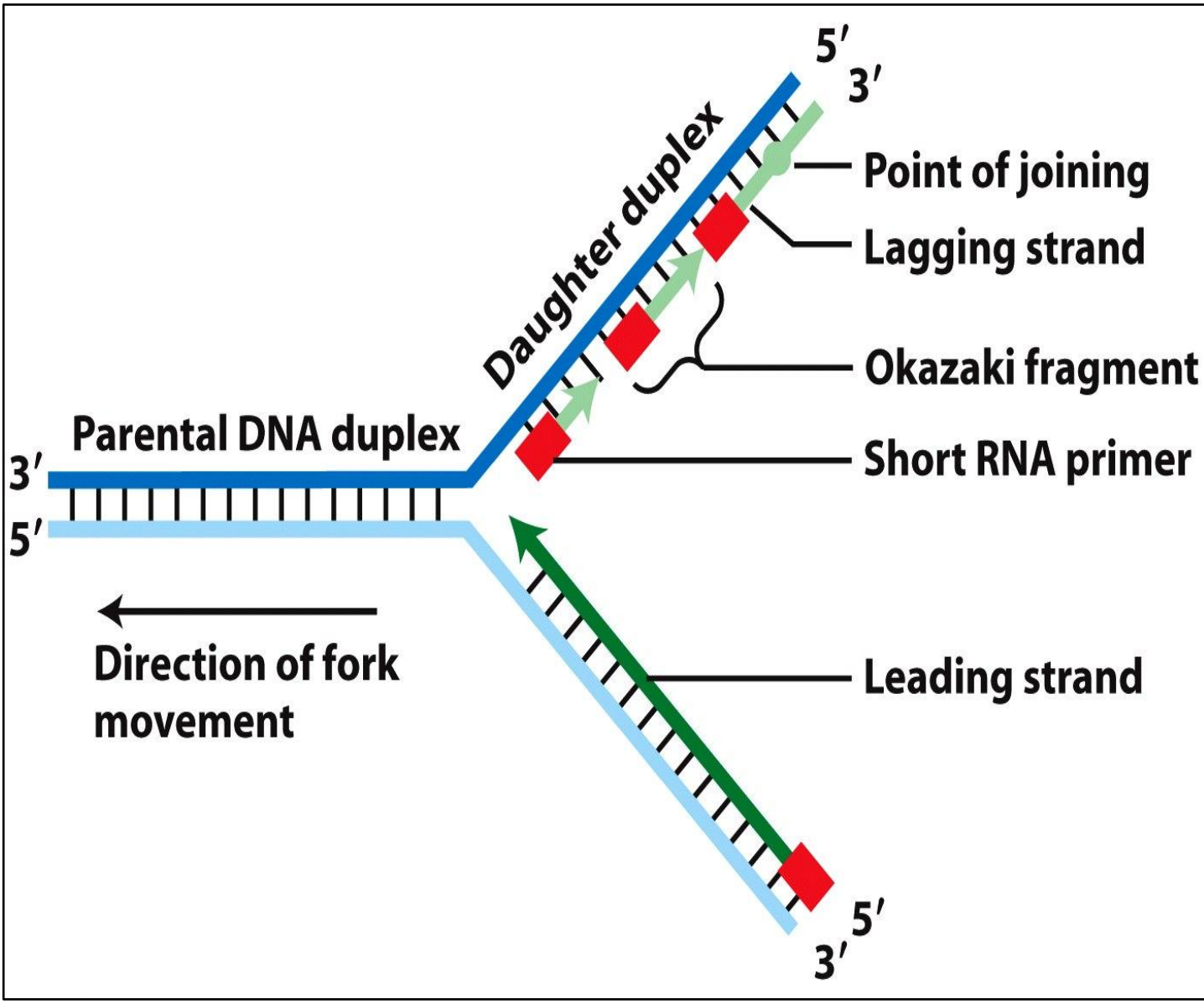
CAF-1 has affinity for PCNA (Proliferating Cell Nuclear Antigen)

CAF-1 associates with PCNA and assembles H3.H4 preferentially on the PCNA-bound DNA

REPLICATION AT THE END CHROMOSOME TELOMERE

TELOMERASE CONCEPT




5′
3′
Daughter duplex
Point of joining
Lagging strand
Okazaki fragment
Parental DNA duplex
Short RNA primer
3′
5′
Direction of fork
movement
Leading strand
5′
3′

Molecular solutions –

1. Become circular during replication and post-replication specific cuts converts back to linear. Example - T_4 phage, λ phage

2. Use a protein - binds to the lagging strand template and uses an amino acid to provide the free 3'OH. Example - Adenovirus

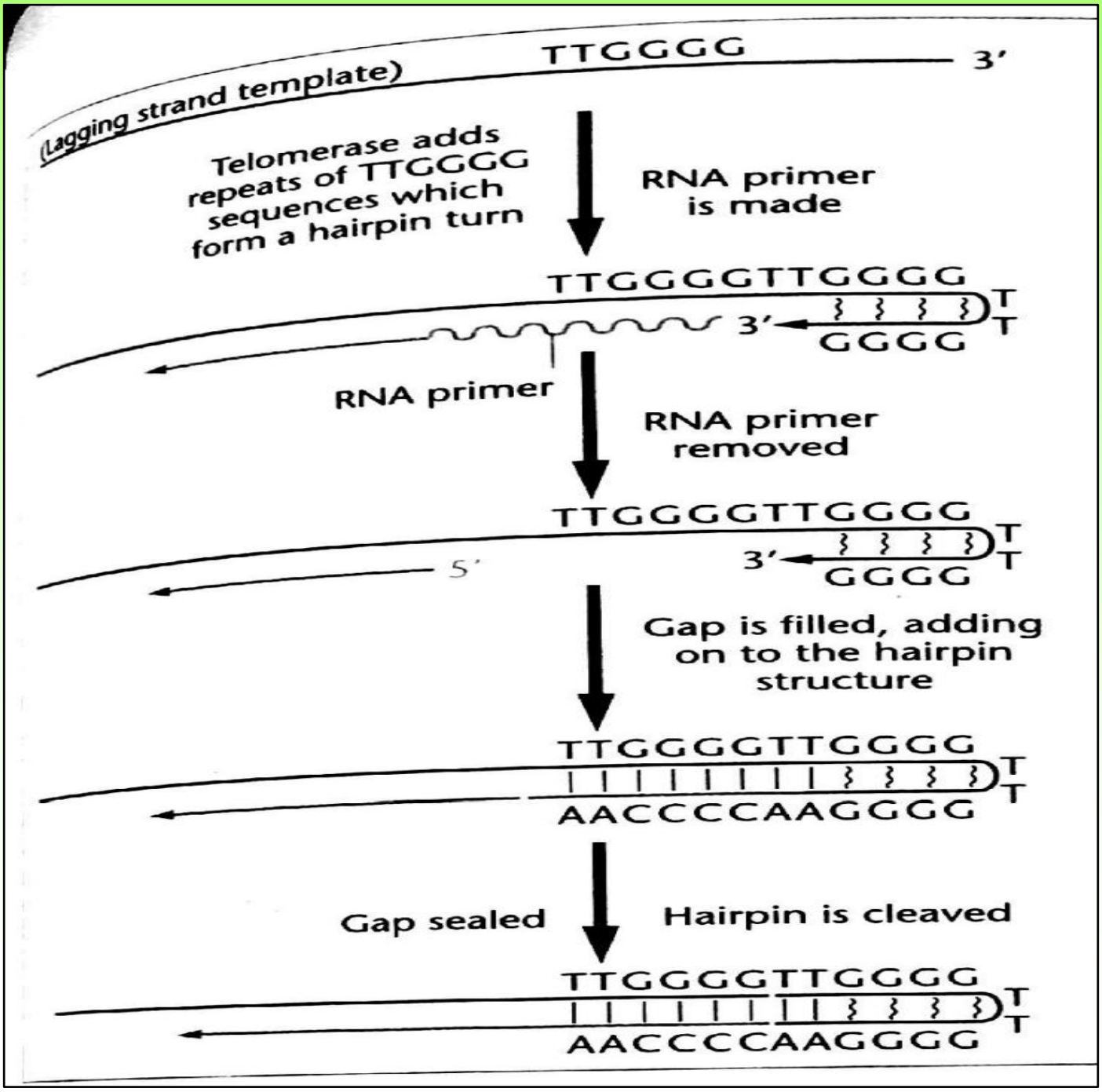
3. Telomeric sequences replicated by telomerase- extend the 3' end of the chromosome, using the repeat sequence of its own RNA. Example - Most eukaryotic cells

Telomeres composed of G-rich tandem repeats (e.g. - in Tetrahymena - TTGGGG, in human being - TTAGGG) and 12-16 base overhang at the 3' end of the chromosome which extends beyond the 5' end of complementary strand.

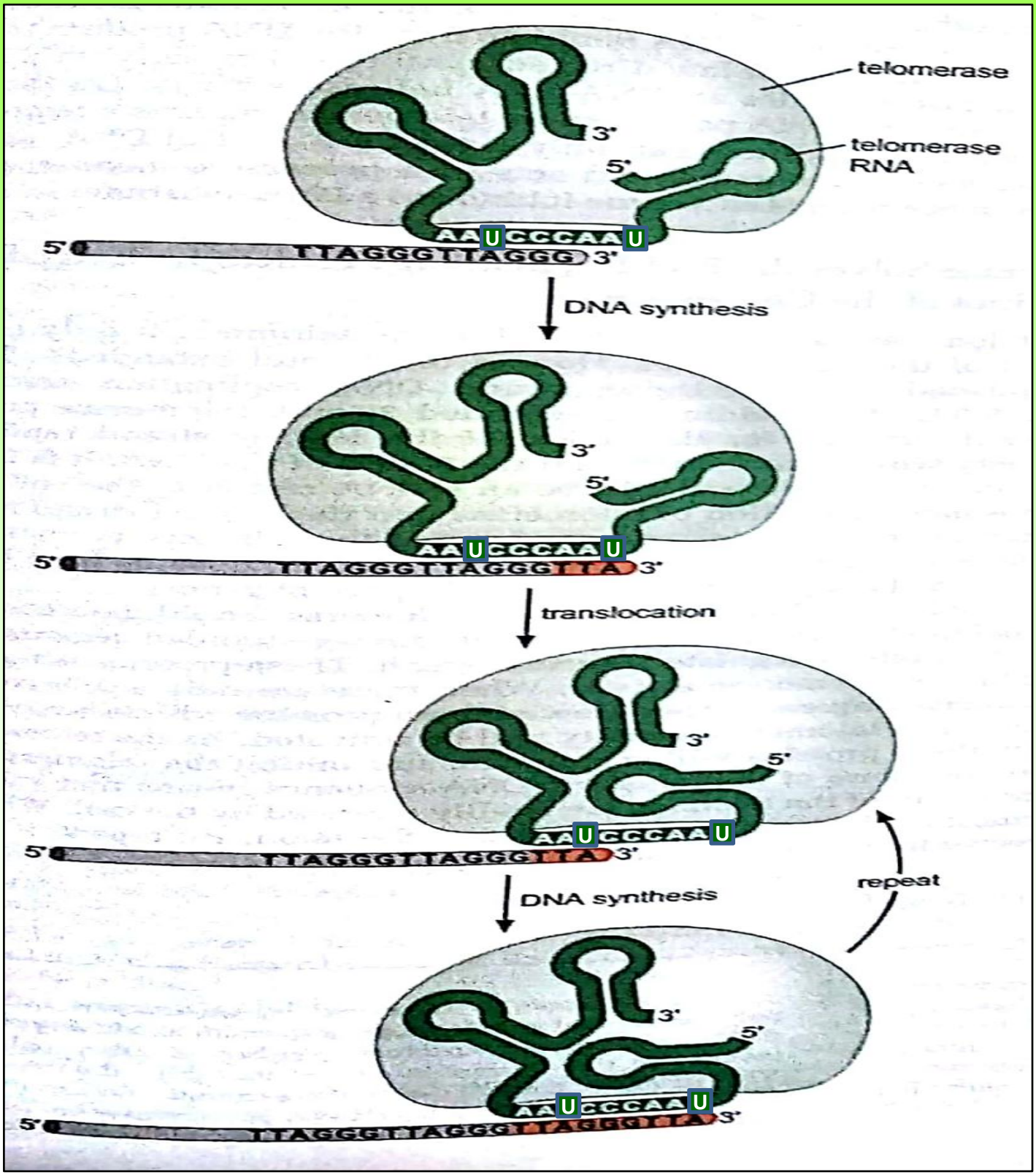
Telomerase - reverse transcriptase enzyme, composed of multiple protein subunits and a RNA component, i.e., it is a ribonucleoprotein.

Using the repeat sequence of its own RNA molecule as template, telomerase can extend the 3' end of one strand by one repeat unit beyond its original length. This action can be repeated several times to lengthen the telomere.

Telomerase add several copies of repeat sequence it has within its RNA to the 3'end of the lagging strand. These repeats are then form a 'hairpin loop', creating a free 3'-OH group on the end of the hairpin that can serve as a substrate for DNA polymerase I. Loop later cleaved off at its terminus and the potential loss of DNA in each subsequent replication cycle is averted.



Telomerase RNA varies in size (150-1300 bases), includes a short region- anneal to 3' end, creating a primer:template junction. Synthesizes DNA to the end of TER but cannot continue to copy the RNA beyond that point. RNA template disengages, reanneals to last 4 nucleotides, repeats this process.



Telomere binding proteins regulate telomerase activity & length

Proteins bound act as weak inhibitors.

Few copies of the repeat, few proteins bound.

As becomes longer, more proteins accumulate and inhibit extension.

This simple negative-feedback loop mechanism (longer telomeres inhibit telomerase) is a robust method to maintain a similar telomere length at the ends of all chromosomes.

Proteins can also modulate telomerase activity

In yeast, Cdc13 protein binds telomere & recruits telomerase - positive activator

In human POT1 protein, acts in the opposite manner – an inhibitor

Telomere-binding proteins also play a crucial role in protecting the ends, simply by coating the telomere