DNA POLYMERASES

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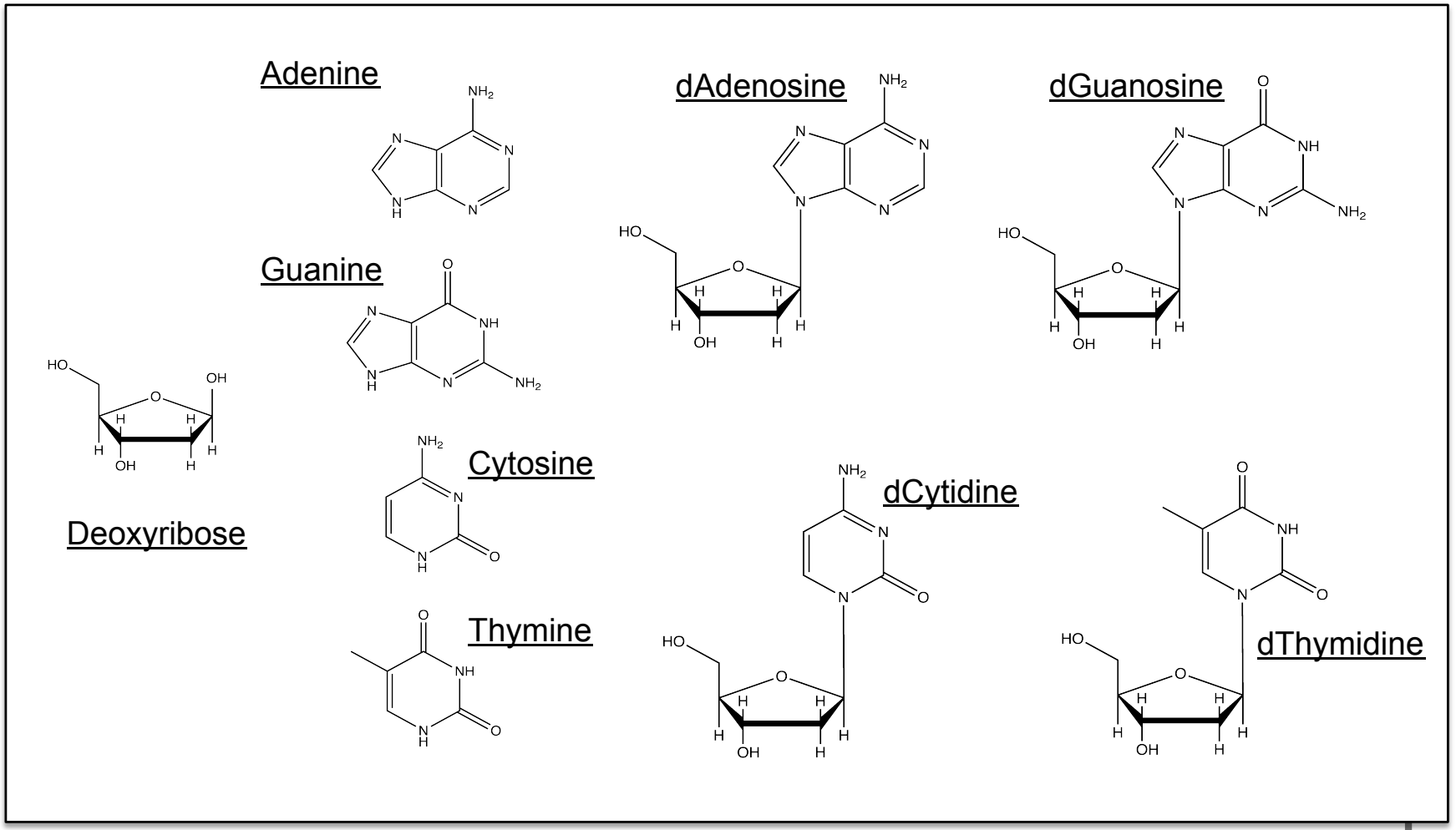
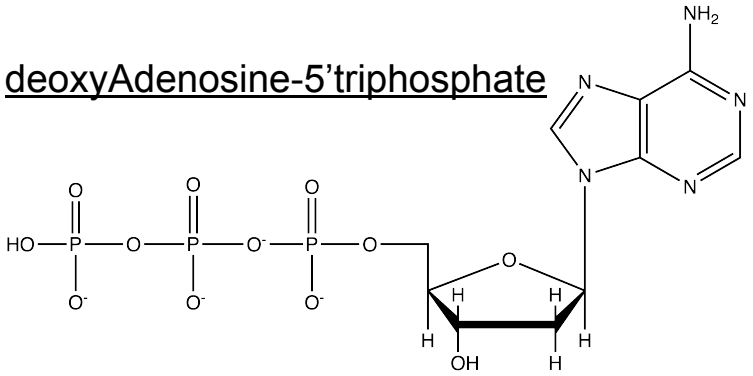
DNA POLYMERASES

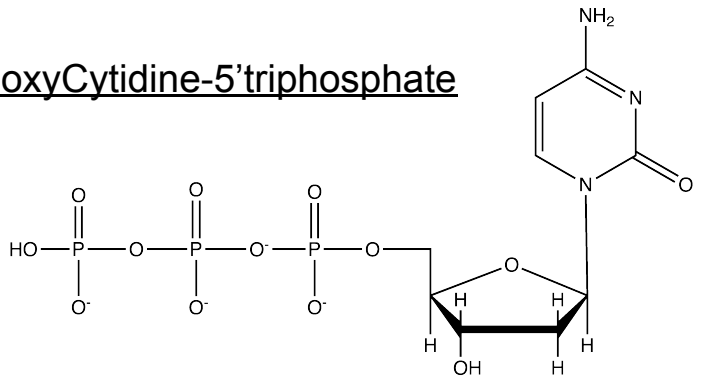


DNA POLYMERASES

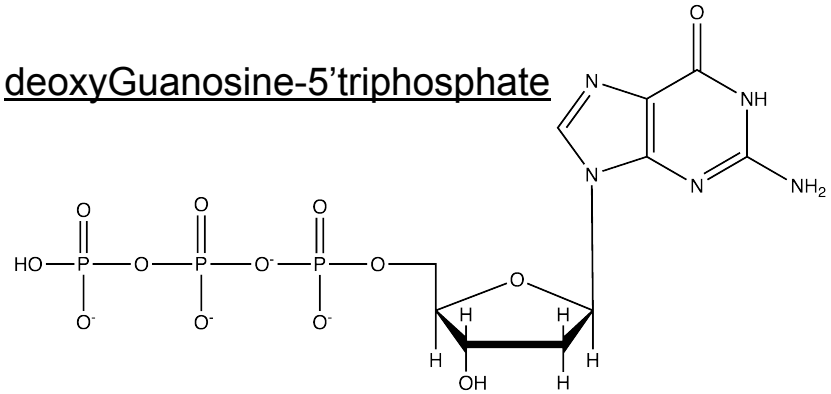
deoxyAdenosine-5'triphosphate



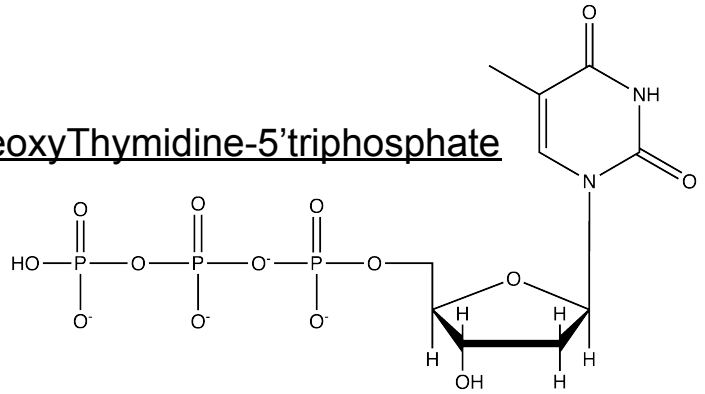
deoxyCytidine-5'triphosphate



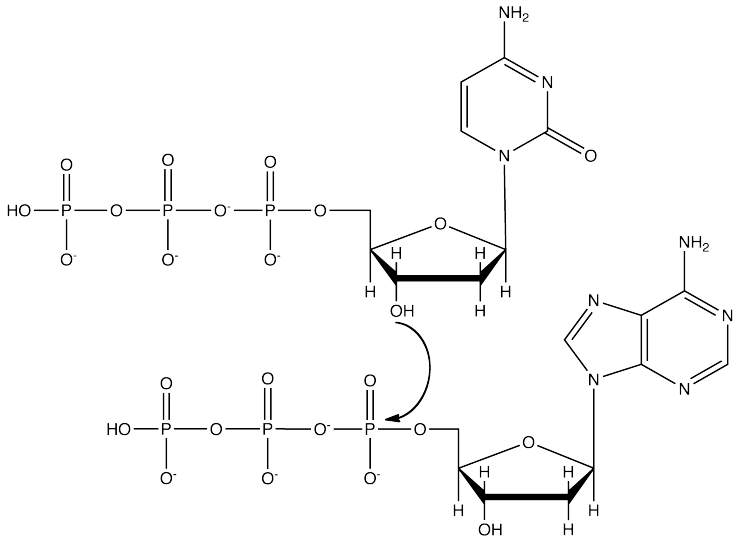
deoxyGuanosine-5'triphosphate



deoxyThymidine-5'triphosphate

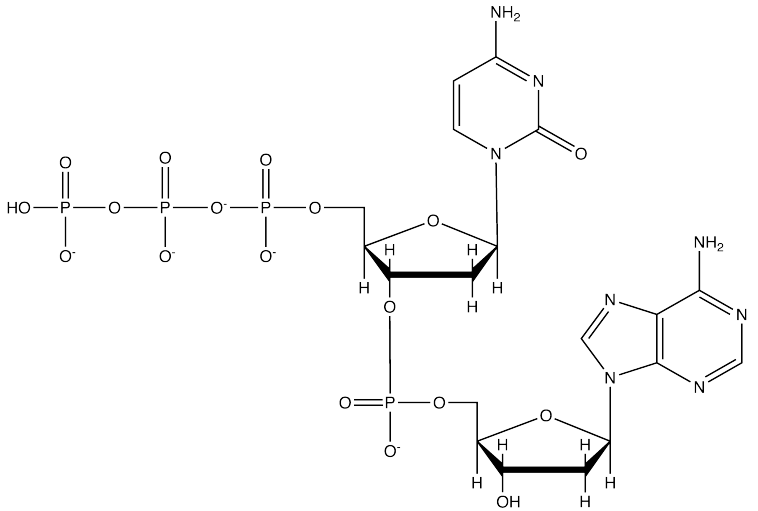


DNA POLYMERASES



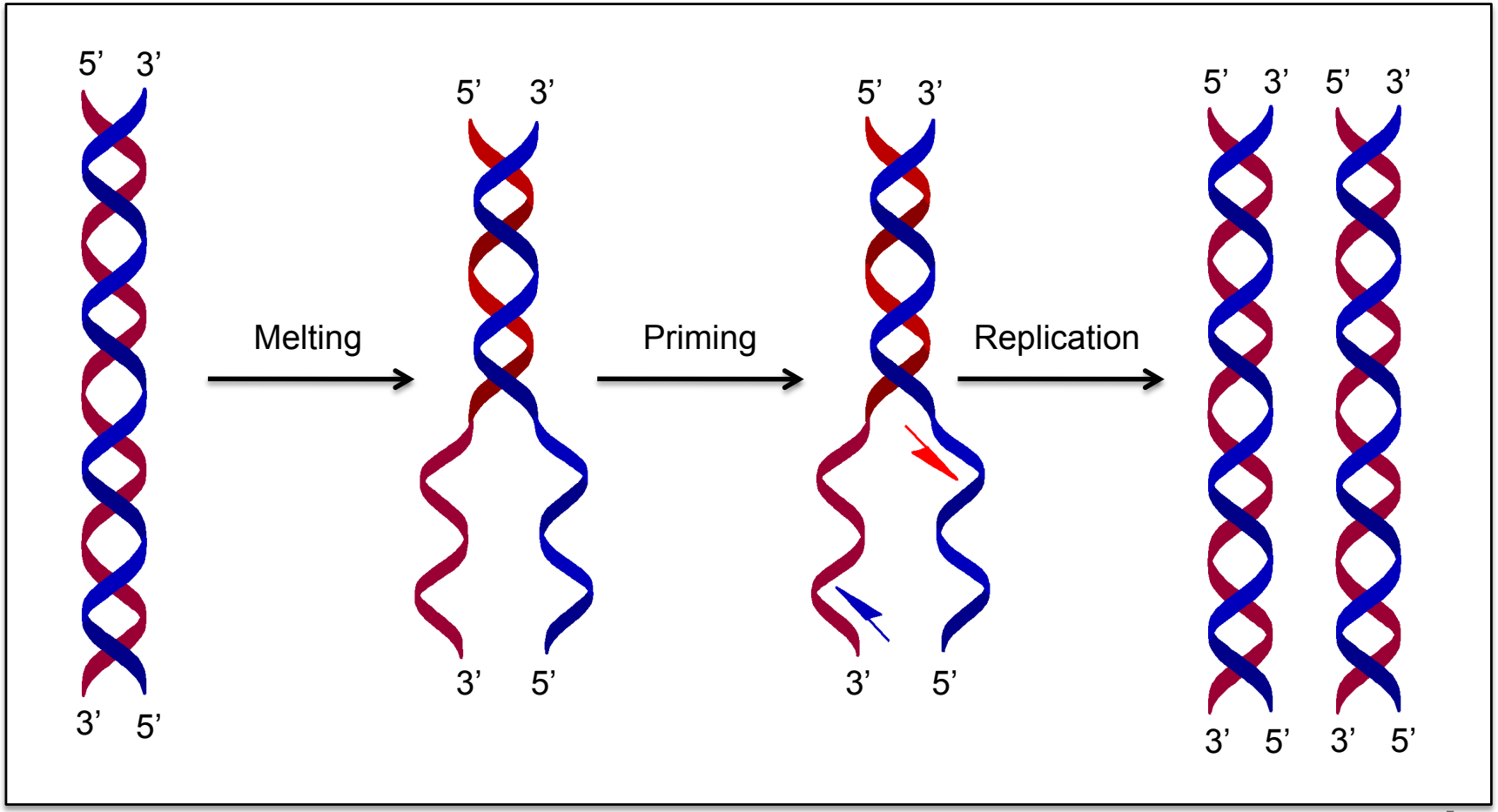
Deoxynucleotides-5'triphosphate are the precursors of DNA. They are obtained from the corresponding deoxynucleotides-5'diphosphates which, in turn, are synthesized from the analogous monophosphates. Occurrence of 3'triphosphates in vivo?

The 3' hydroxyl nucleophilically attacks the α phosphate to yield a phosphodiester bond and pyrophosphate (PPi). DNA Polymerases are enzymes catalyzing this reaction. They need DNA template, dNTPs, and a primer. They do not catalyze de novo synthesis of DNA.

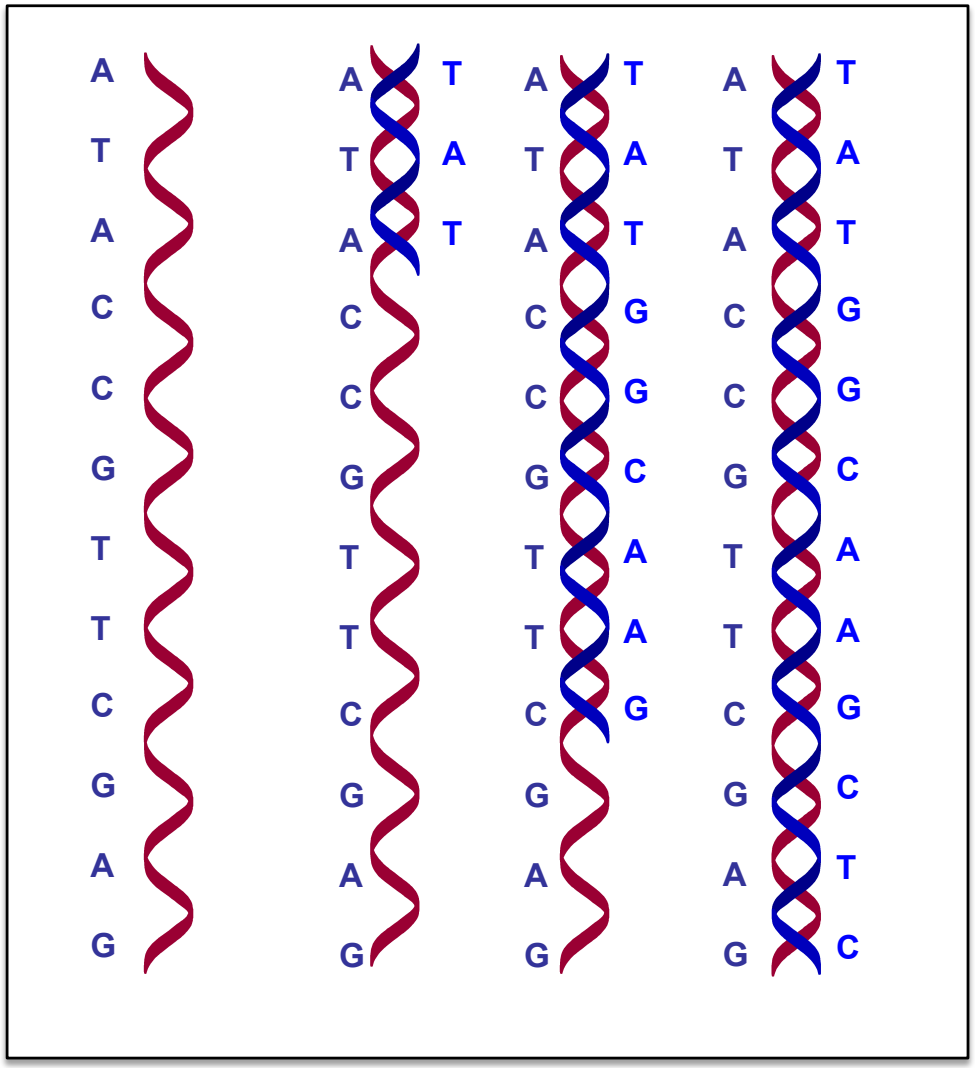


DNA POLYMERASES

Direction of replication is always 5' to 3'



DNA POLYMERASES



Template DNA strand:
3'-ATACCGTTCGAG-5'

Primer:
5'-TAT-3'

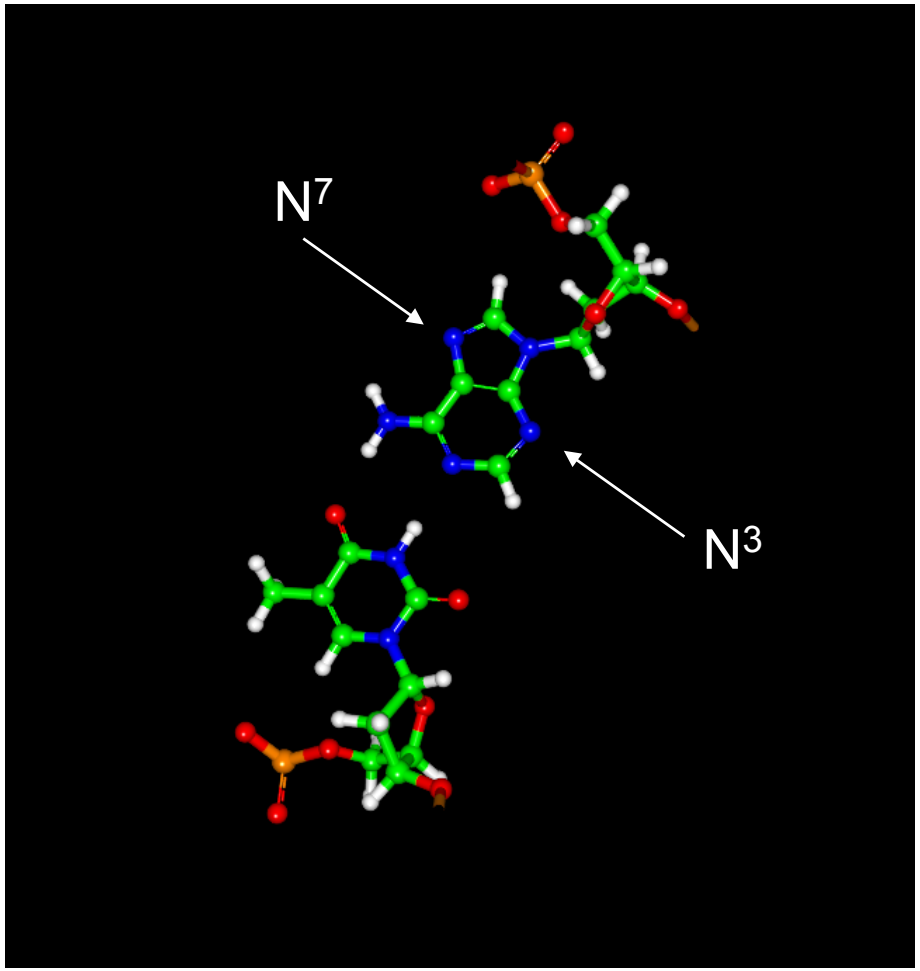
3'-ATACCGTTCGAG-5'
5'-TAT-3'

3'-ATACCGTTCGAG-5'
5'-TAT-3'

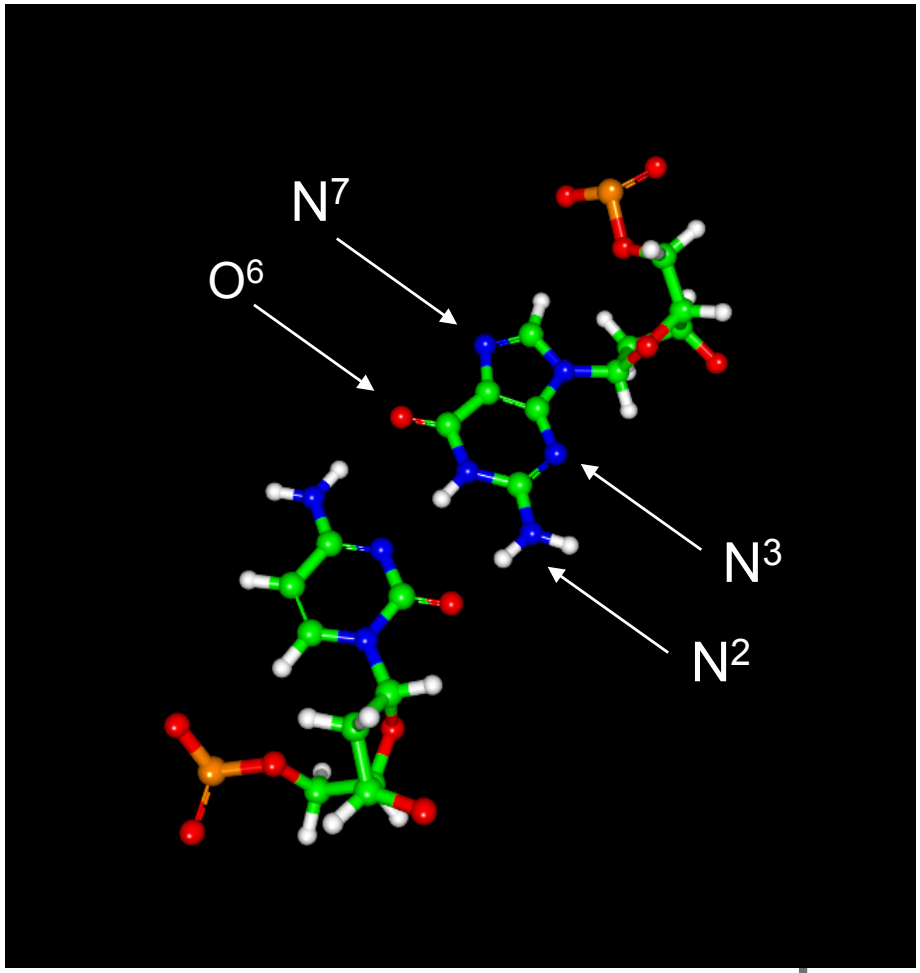
Direction of replication is 5' to 3'
No DNA Polymerase behaves 3' to 5'.

DNA POLYMERASES

Adenine-Thymine

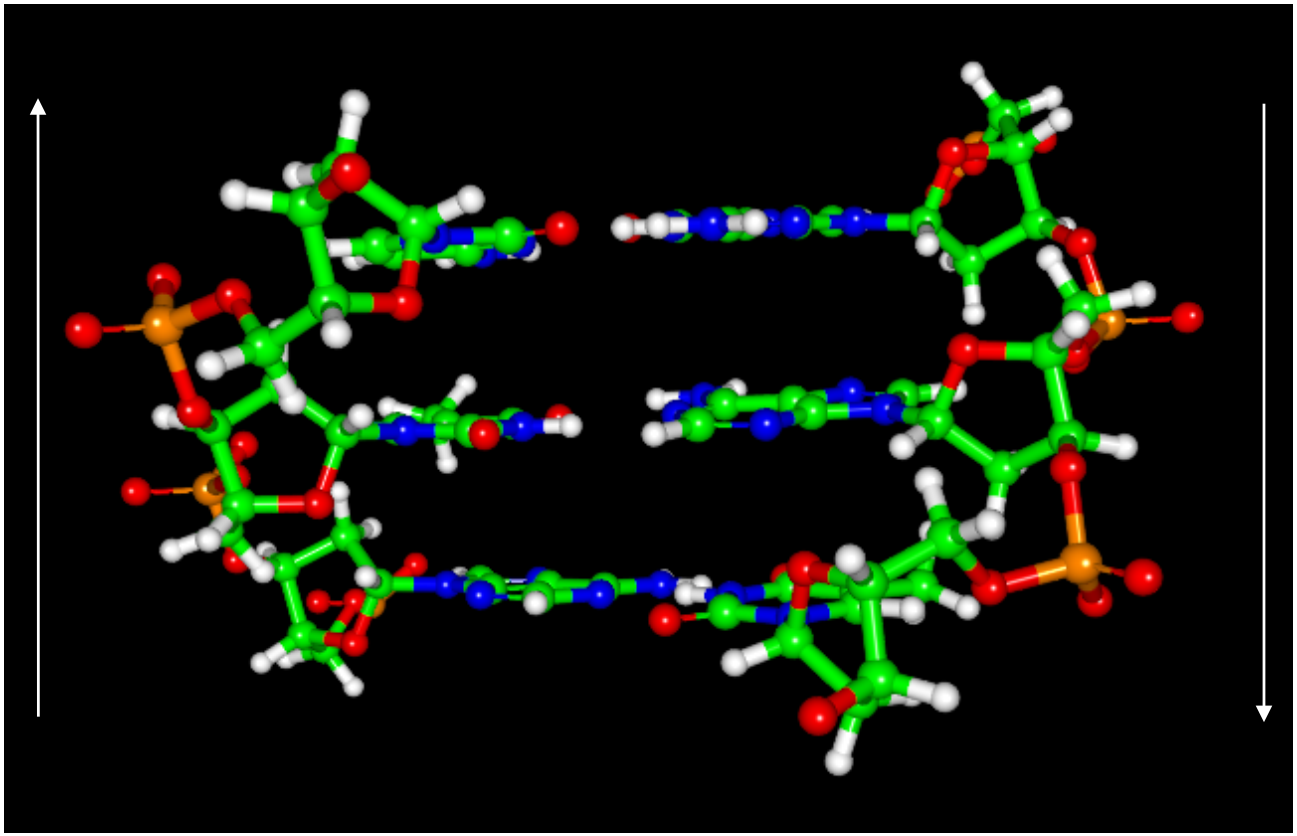


Guanine-Cytosine



DNA POLYMERASES

3'end

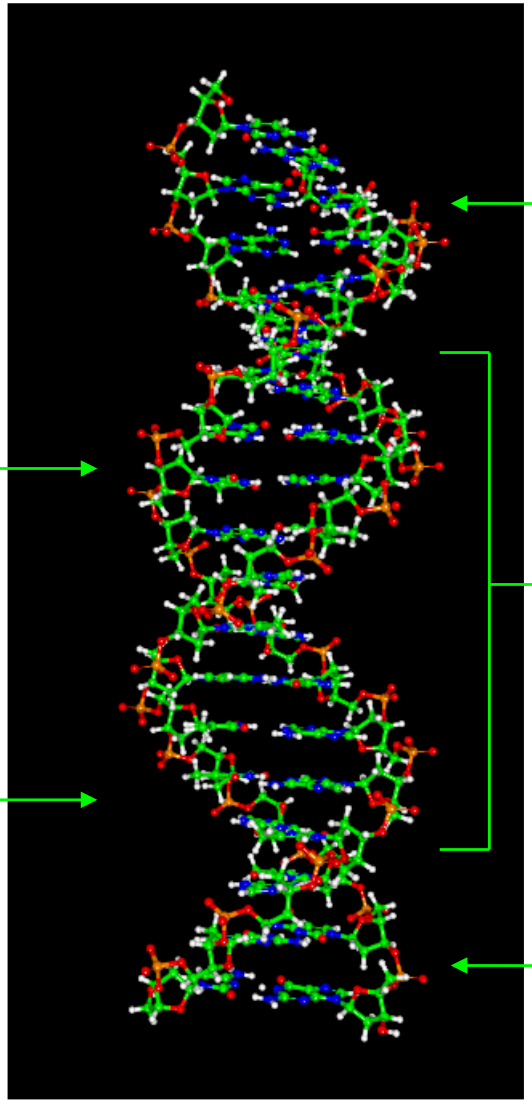


5'end

5'end

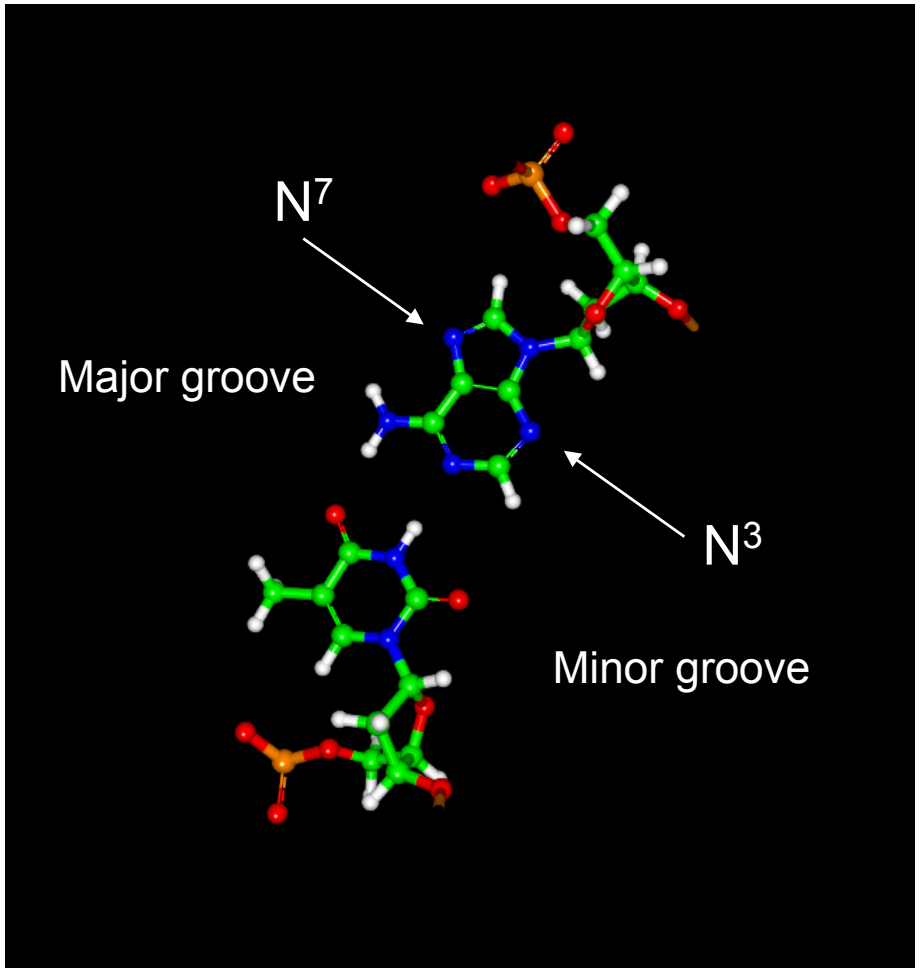
3'end

DNA POLYMERASES

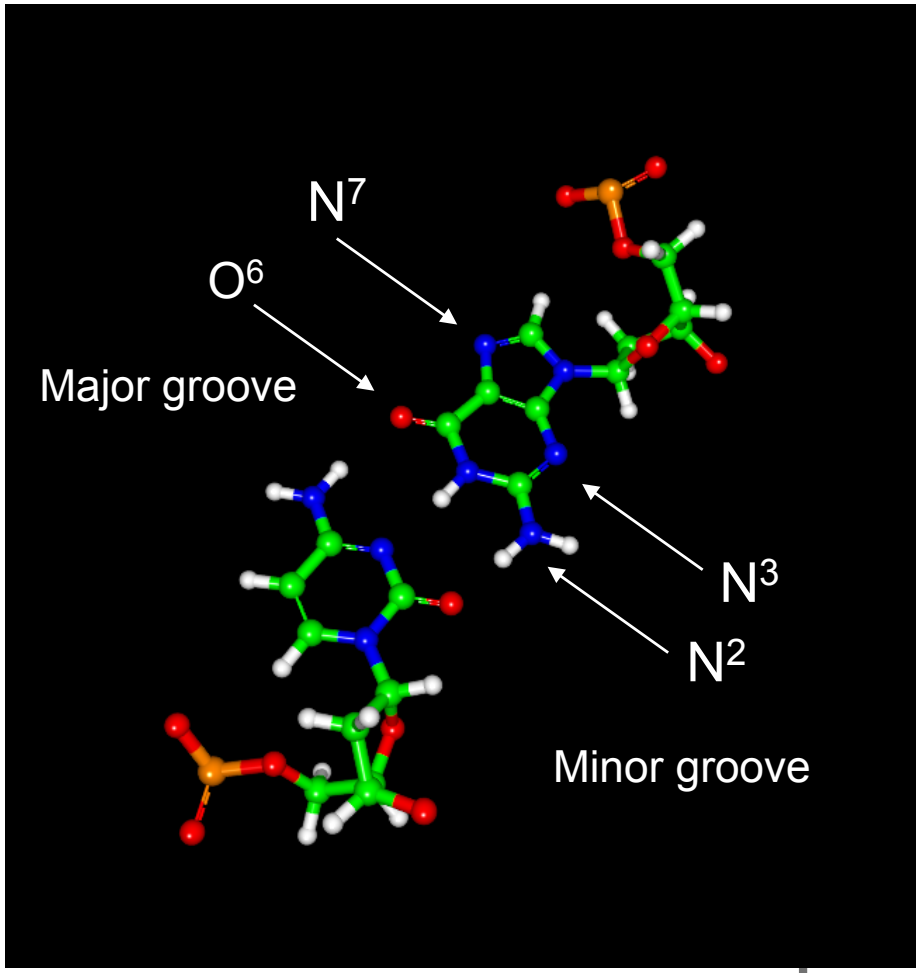


DNA POLYMERASES

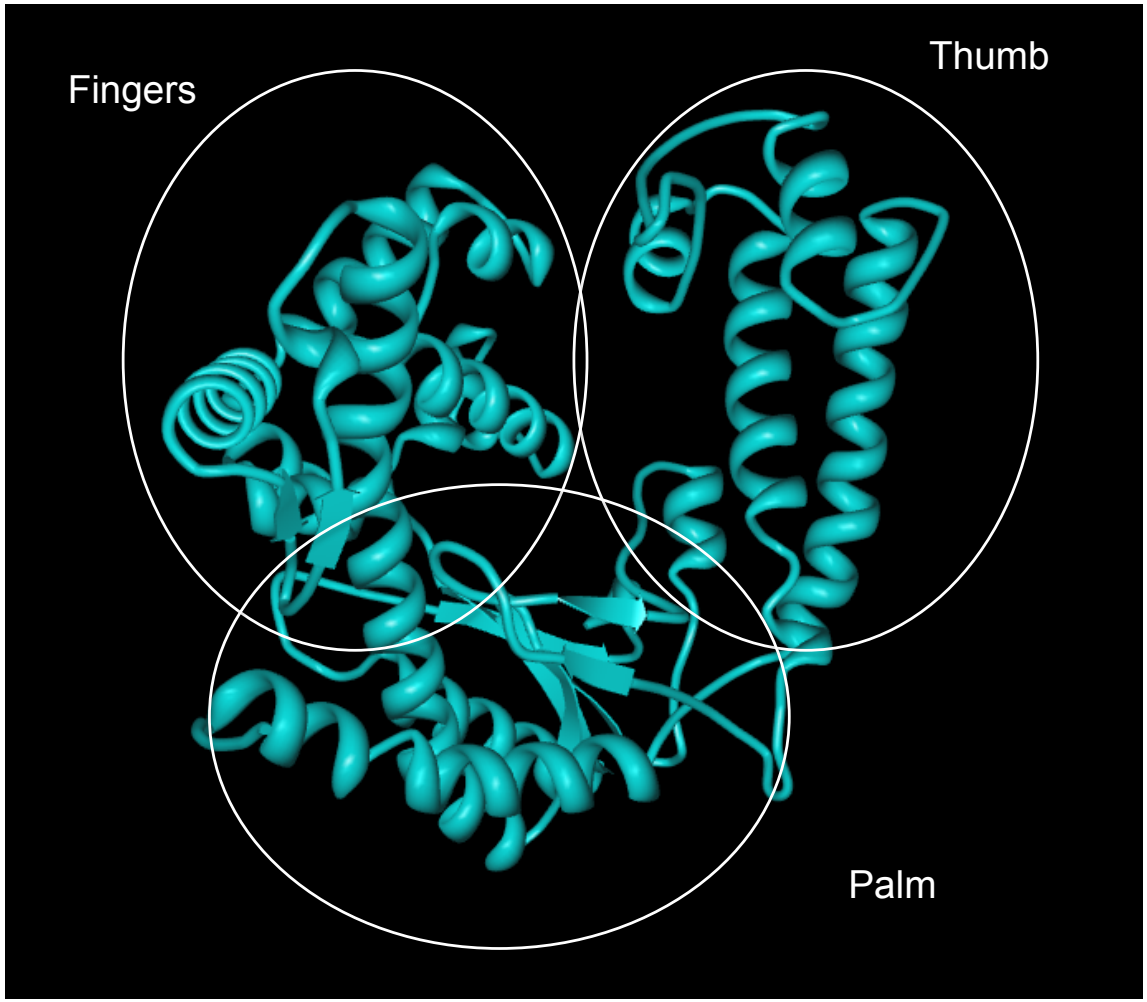
Adenine-Thymine



Guanine-Cytosine



DNA POLYMERASES

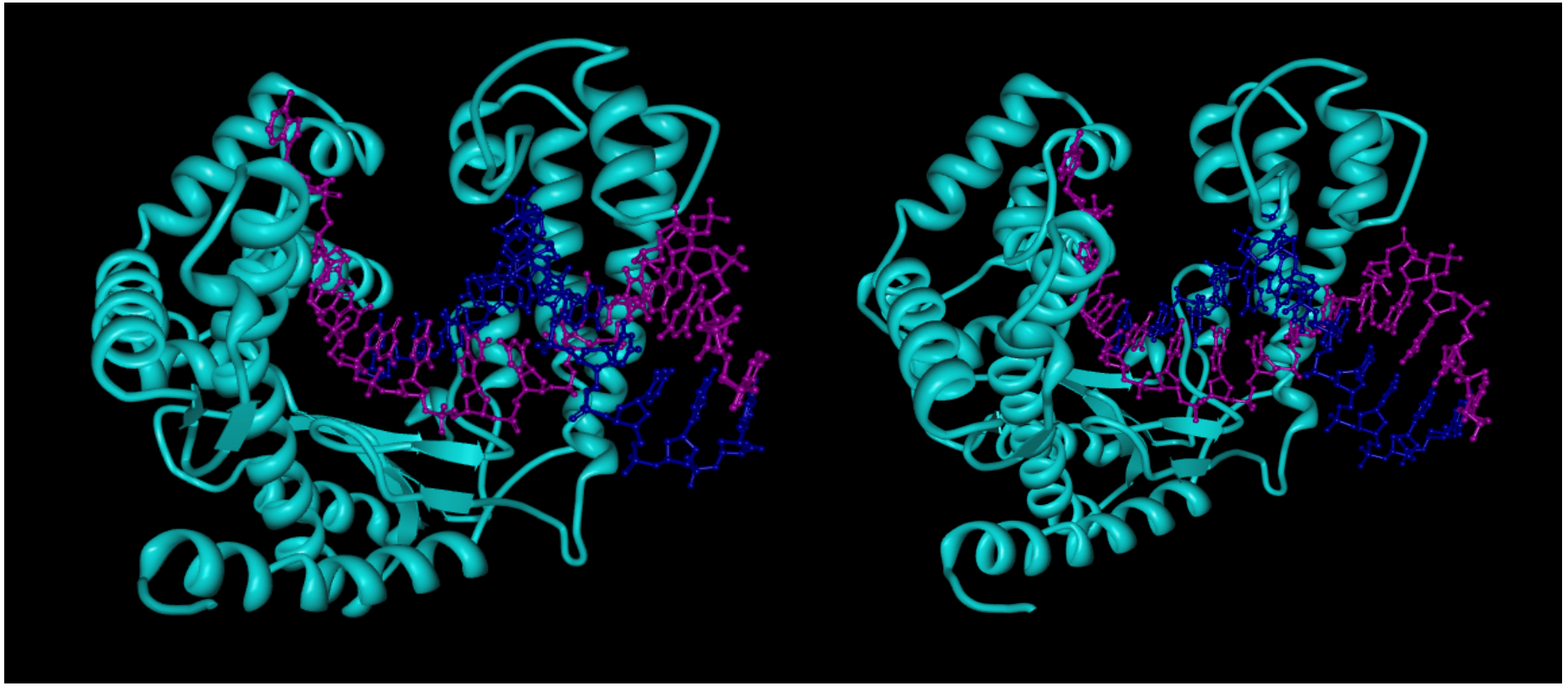


Thermus aquaticus DNA Pol

The shape of the enzyme is similar to a right hand (thumb, palm, and fingers).
All DNA Polymerases feature this architecture.
Conformation of thumb and fingers is essentially alpha.
Palm contains a beta-sheet, with a particular topology.
The active site resides in the palm region.
Incoming deoxynucleotides are bound by the fingers.

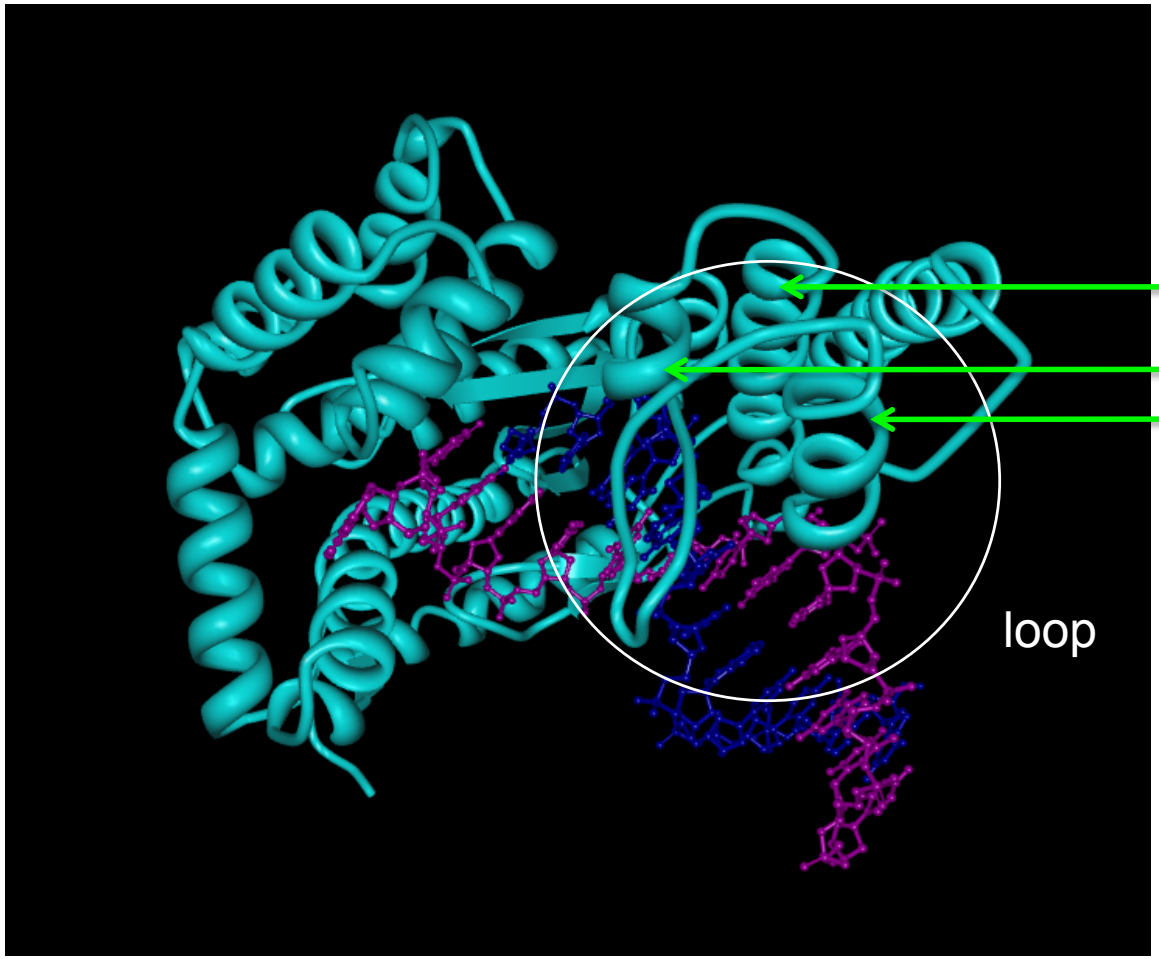
DNA POLYMERASES

Interaction with DNA. Purple: template. Blue: primer.



Double stranded DNA interacts with palm. The single stranded DNA with fingers.

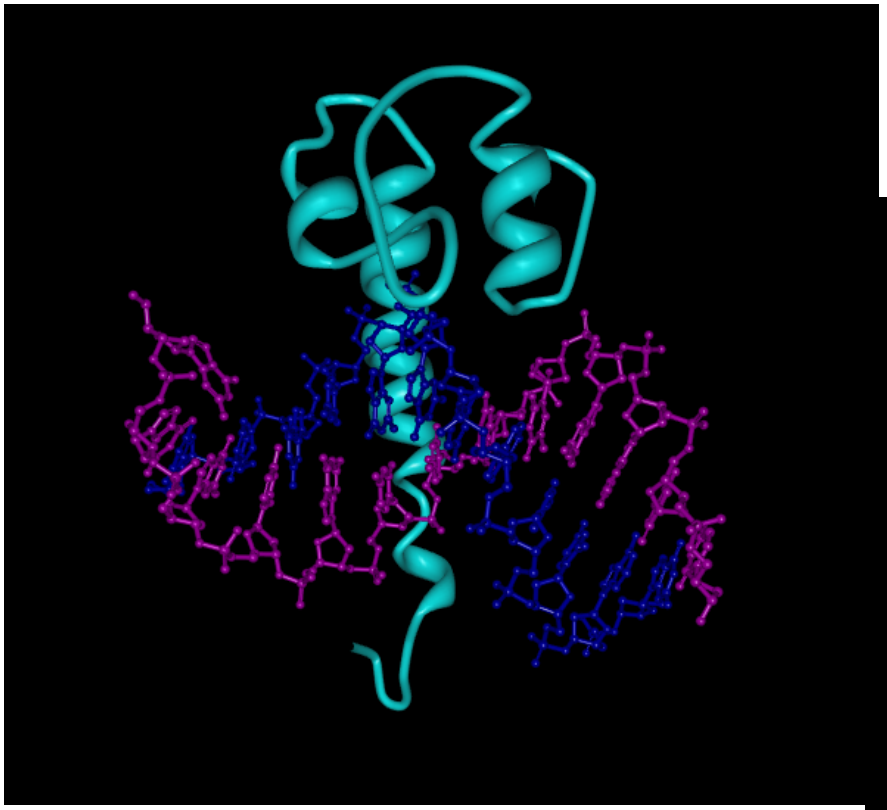
DNA POLYMERASES



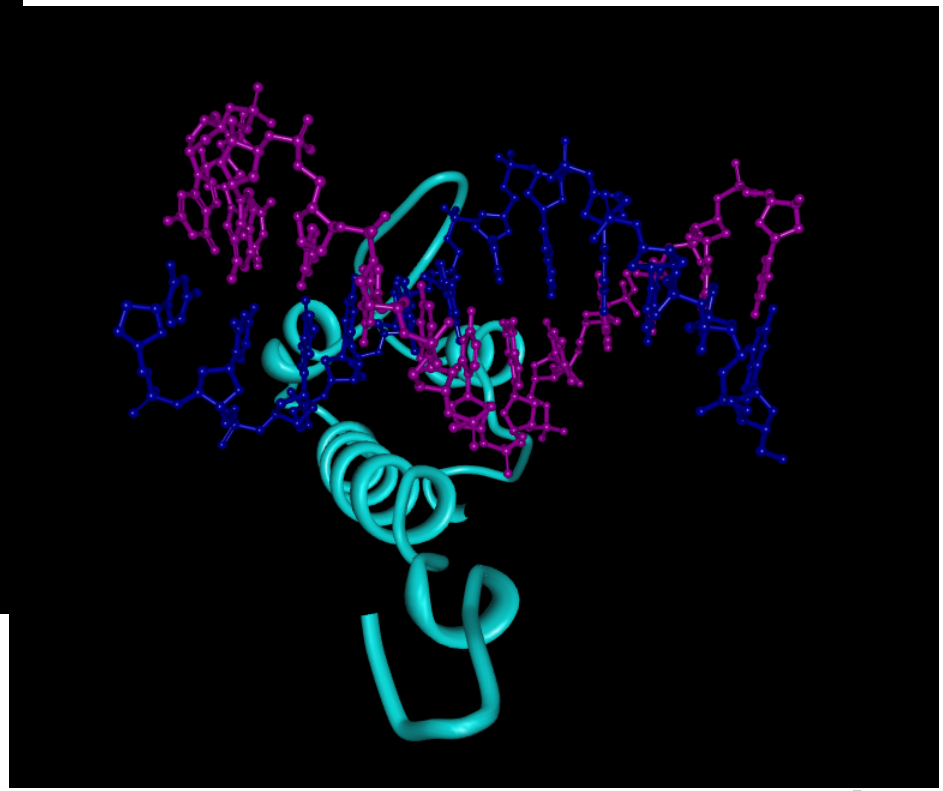
Double-stranded DNA is encircled by the thumb.
DNA-protein contacts:

- alpha helices H1 and H2
- long loop connecting H1 and H2
- alpha helix

DNA POLYMERASES



Amino acids interacting with DNA:
K, R, S, T, N, Q, E



Interactions are mostly between DNA phosphate backbone and protein.

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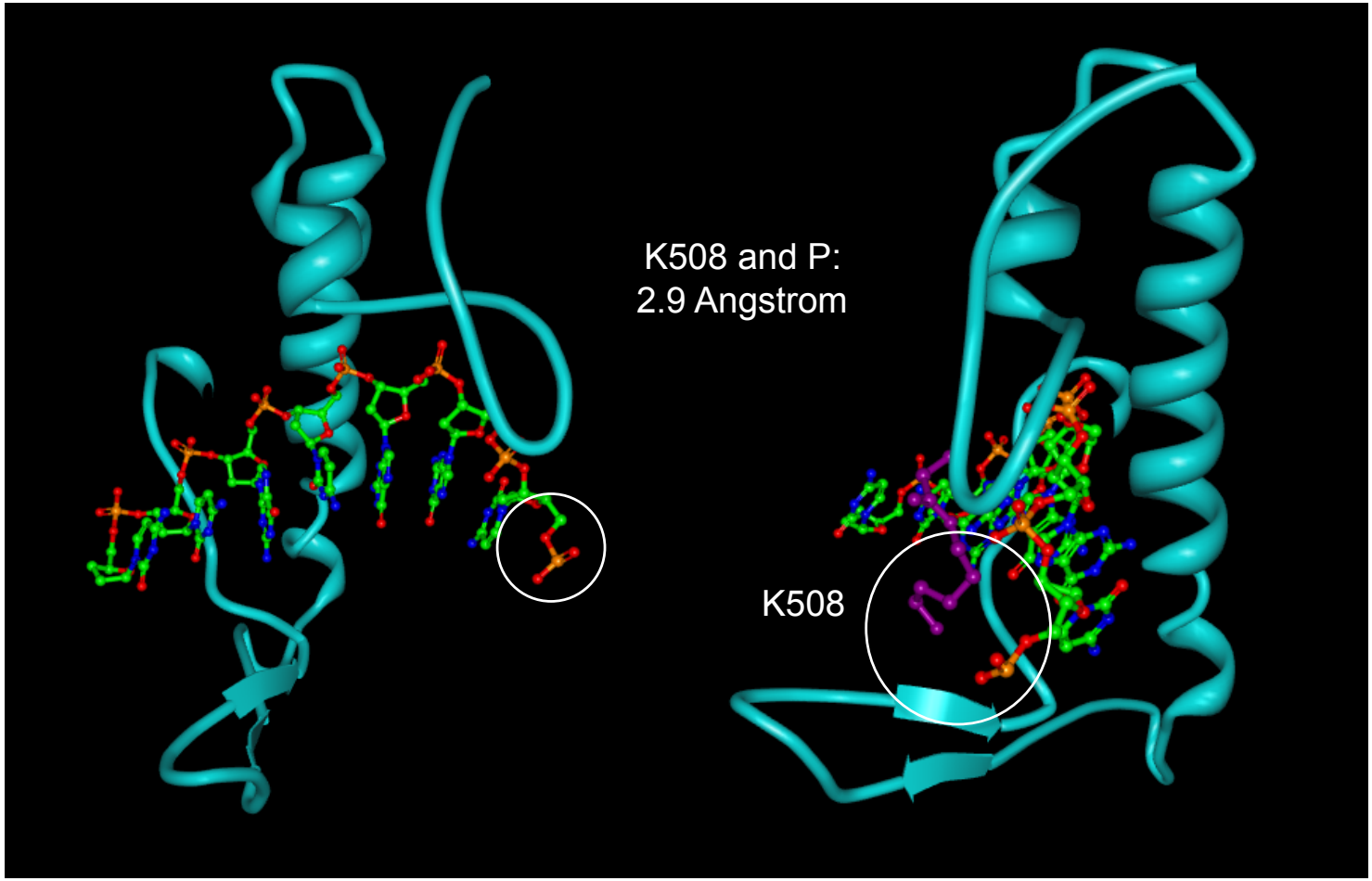
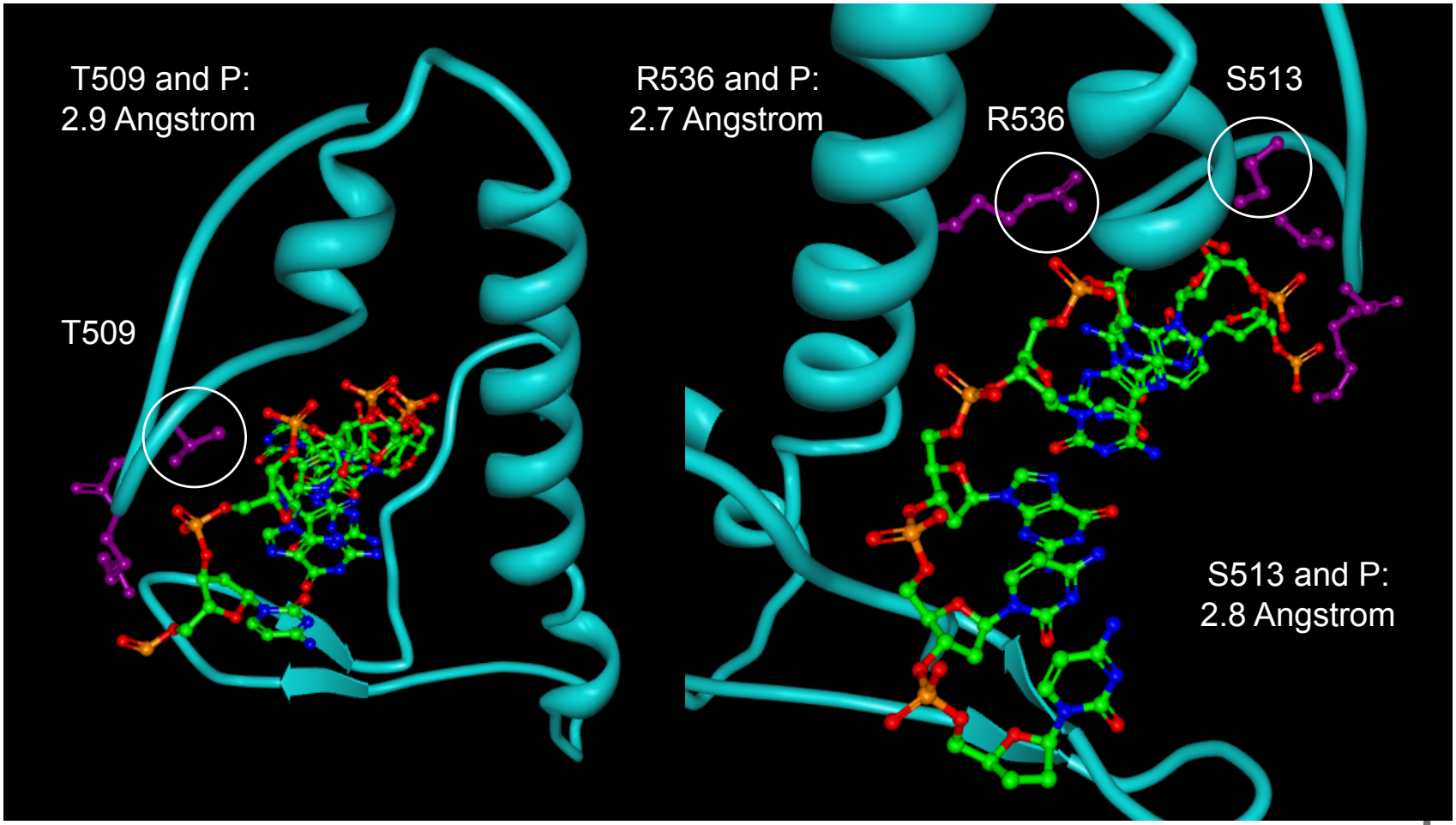
Protein-DNA interactions



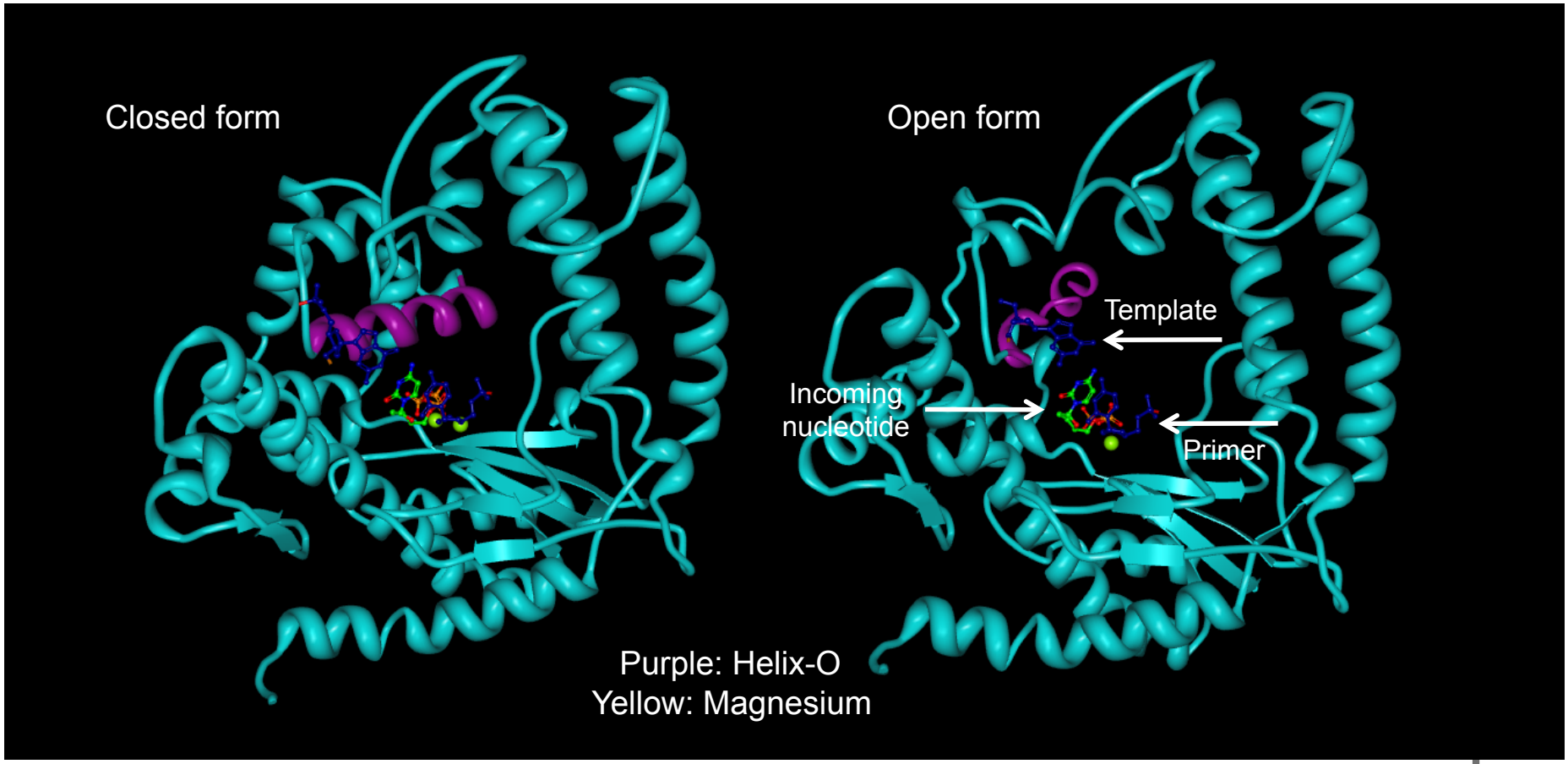
DNA POLYMERASES

Protein-DNA interactions

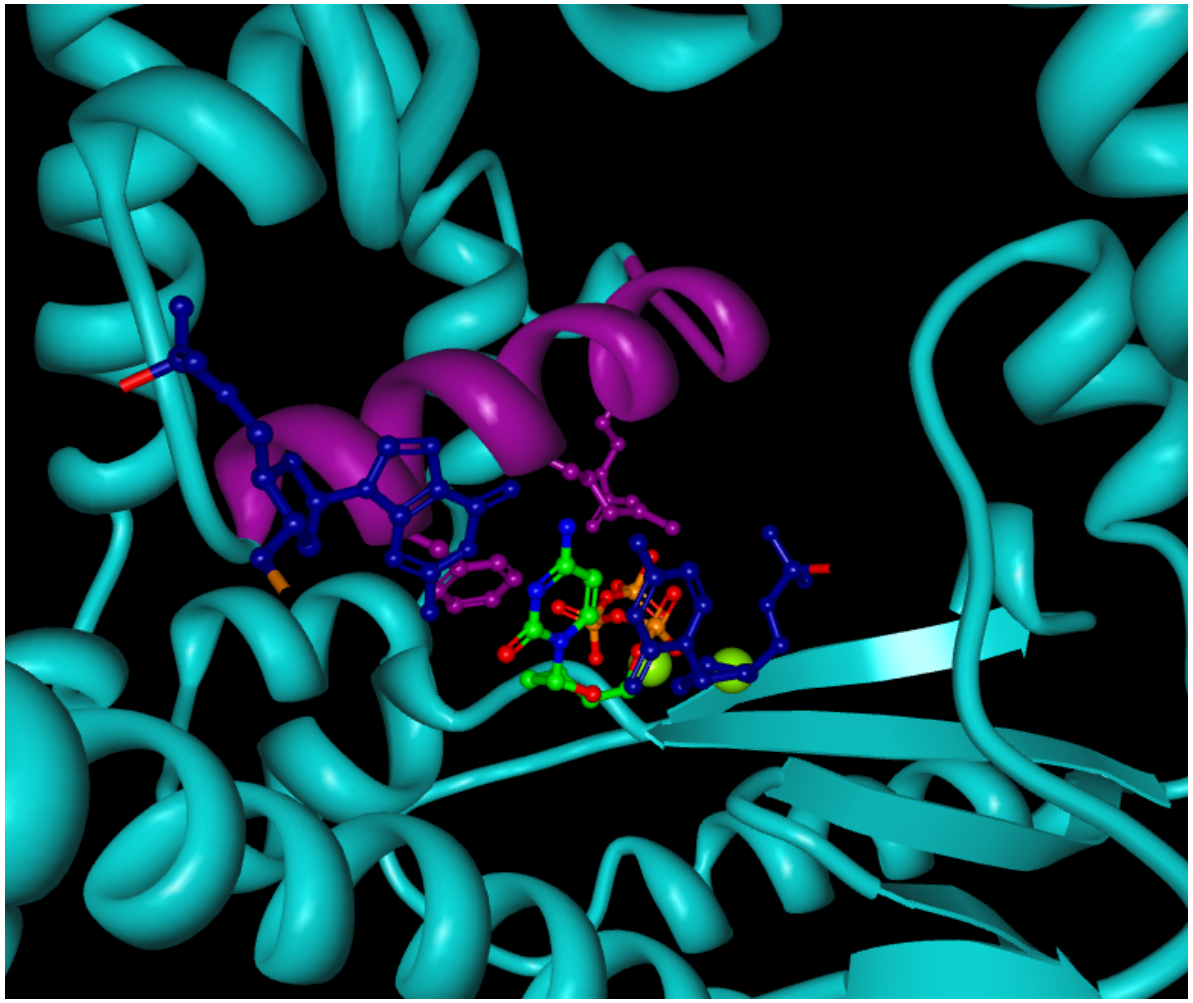


DNA POLYMERASES

Open and closed forms of DNA Polymerase-DNA-nucleotide ternary complex



DNA POLYMERASES



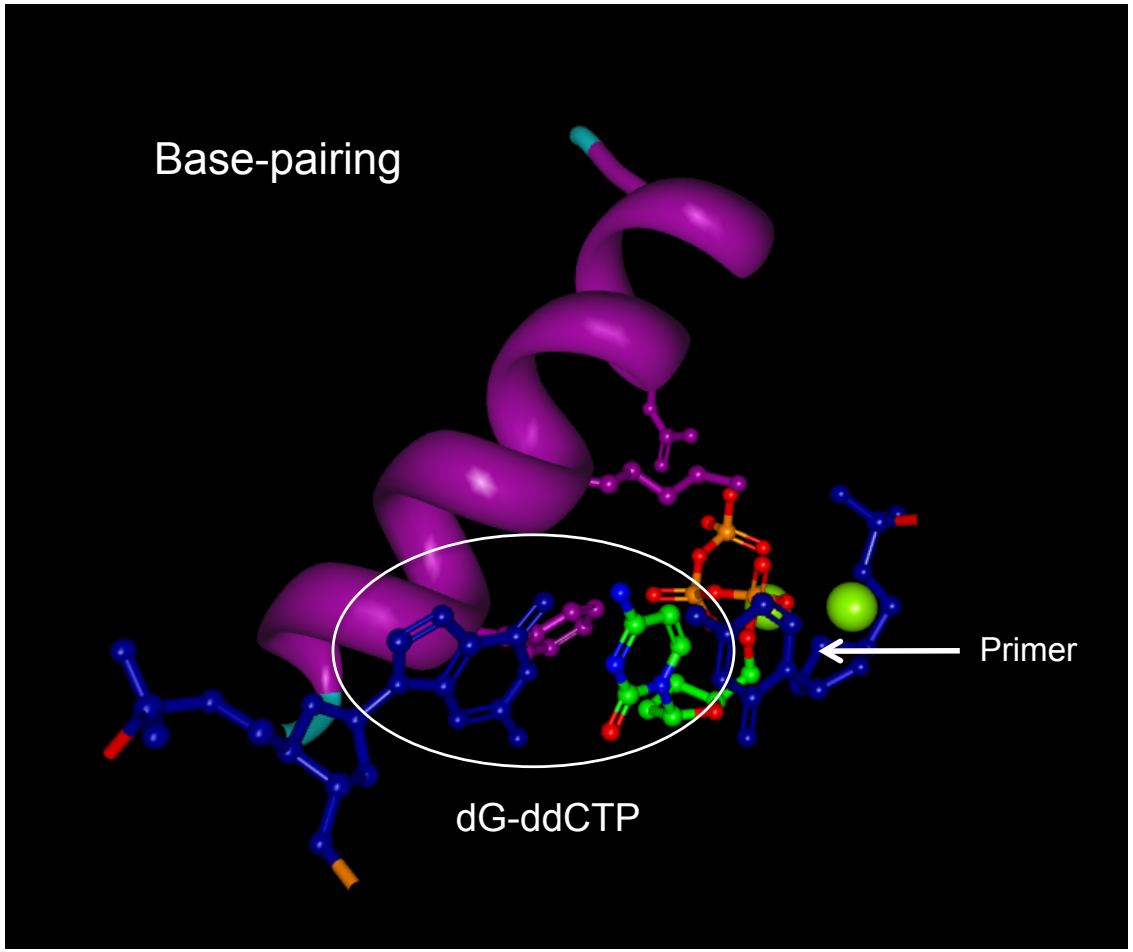
Helix-O in closed form

Interaction with the incoming nt (atom-type coloured). The last nucleotide in the primer is a dideoxy-Cytosine.

- F667 stacks with the base
- K663 interacts with P
- R659 interacts with P

The template base (G) and the incoming nucleotide ddCTP are paired. The enzyme is able to discriminate between Watson-Crick (WC) and non-WC base pairings. This discrimination is a major factor of replication fidelity.

DNA POLYMERASES

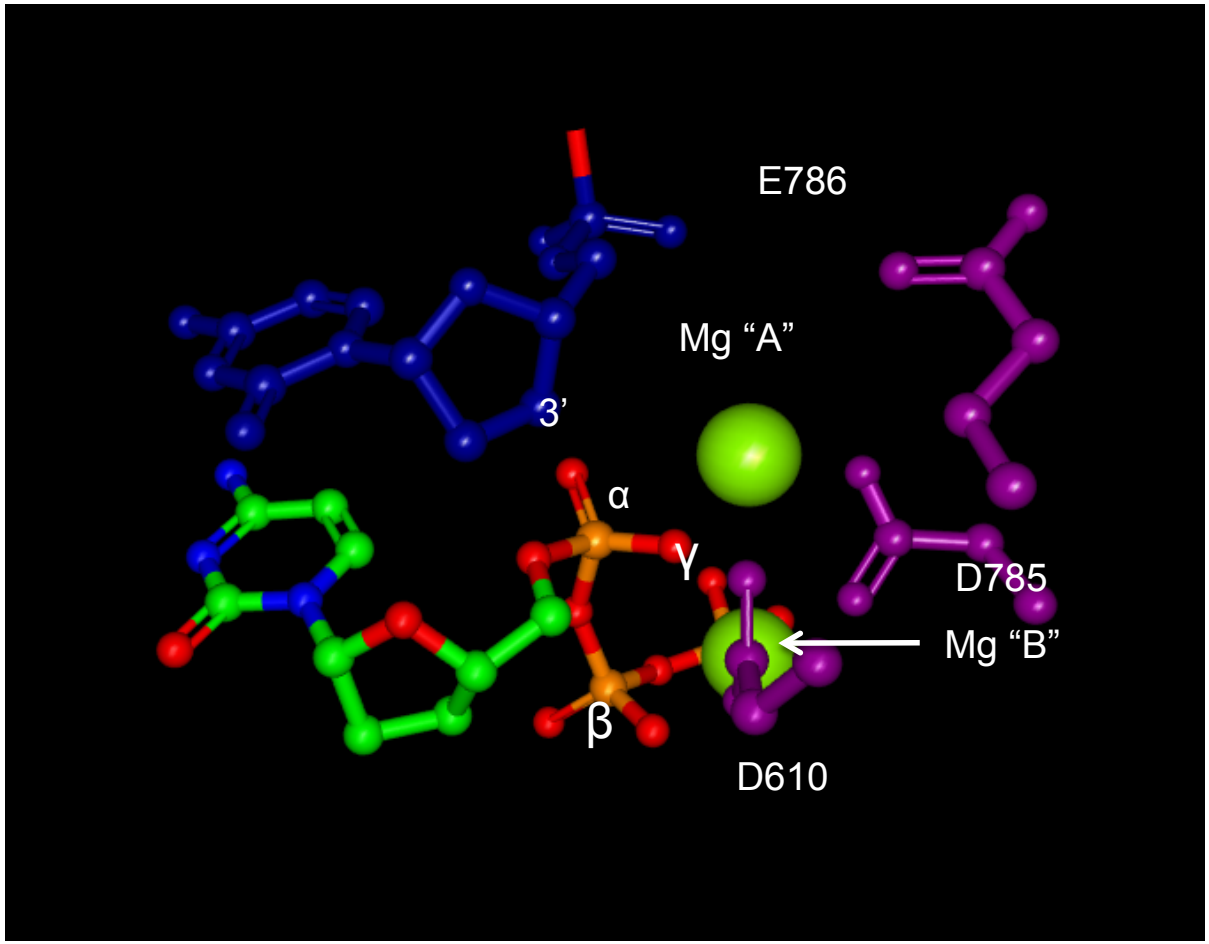


Elongation reaction:
the 3'-OH of the primer attacks (nucleophile) the α-phosphate of the incoming nucleotide.

How can the nucleophilicity of the 3'-OH be increased?
Essential components:
- 2 Magnesium atoms
- 2 Aspartates (Palm)
- 1 Glutamate (Palm)

The acidic residues coordinate the Magnesium atoms. The Mg Atoms are also coordinated to phosphates

DNA POLYMERASES



Mg^{2+} “B” coordinated to:

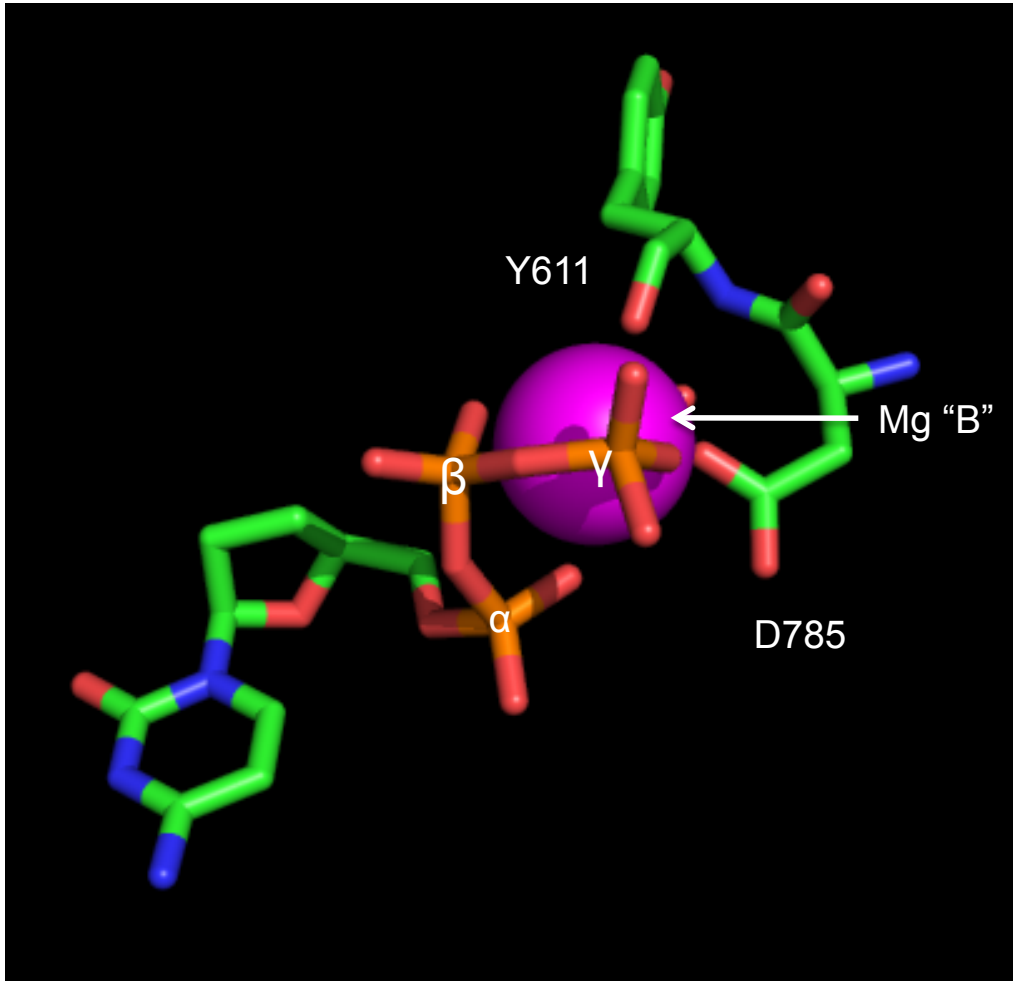
- α, β and γ phosphates
- D610 and D785
- Y611 (not shown)

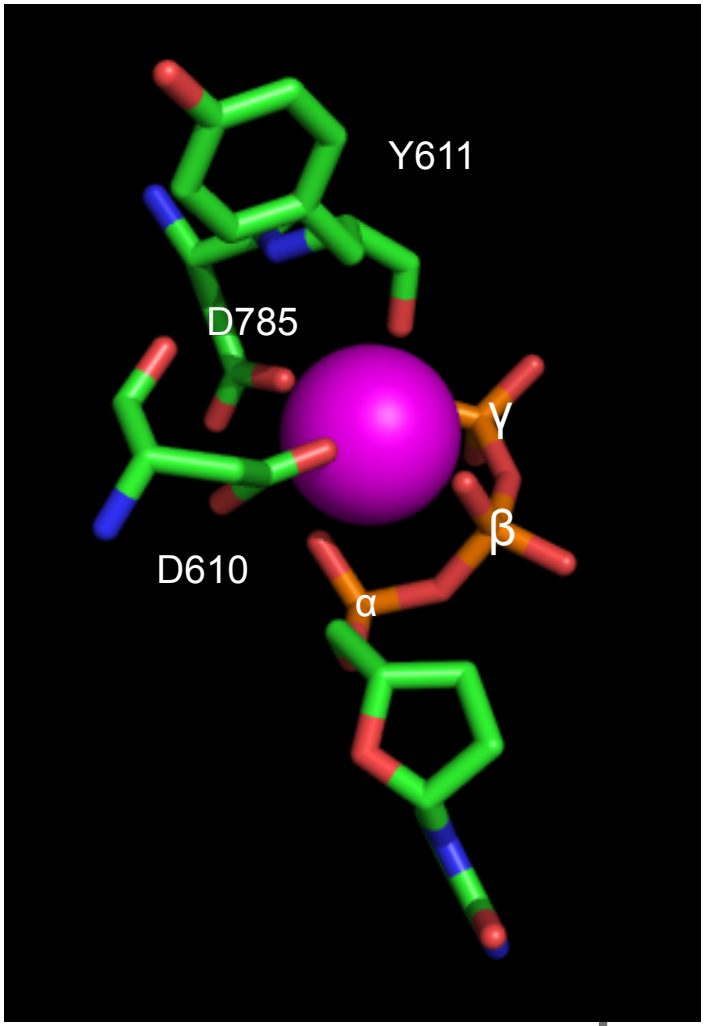
Mg^{2+} “A” coordinated to:

- α phosphate
- D610 and D785
- 2 H_20
- vacancy of 1 site

Mg “A” increases acidity of 3’-OH

DNA POLYMERASES





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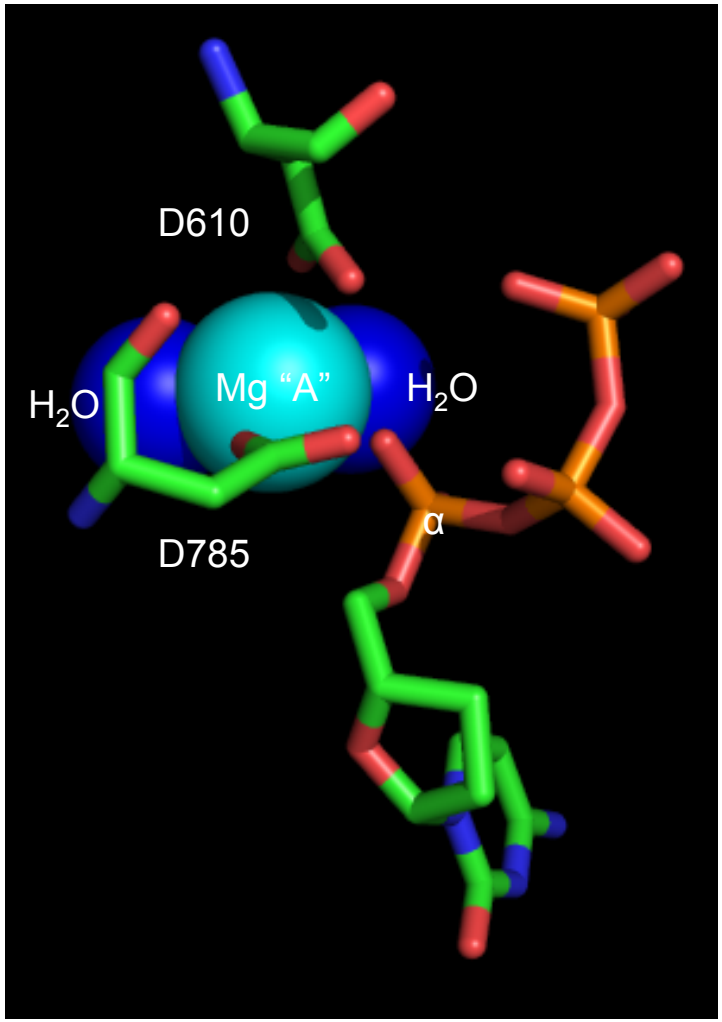
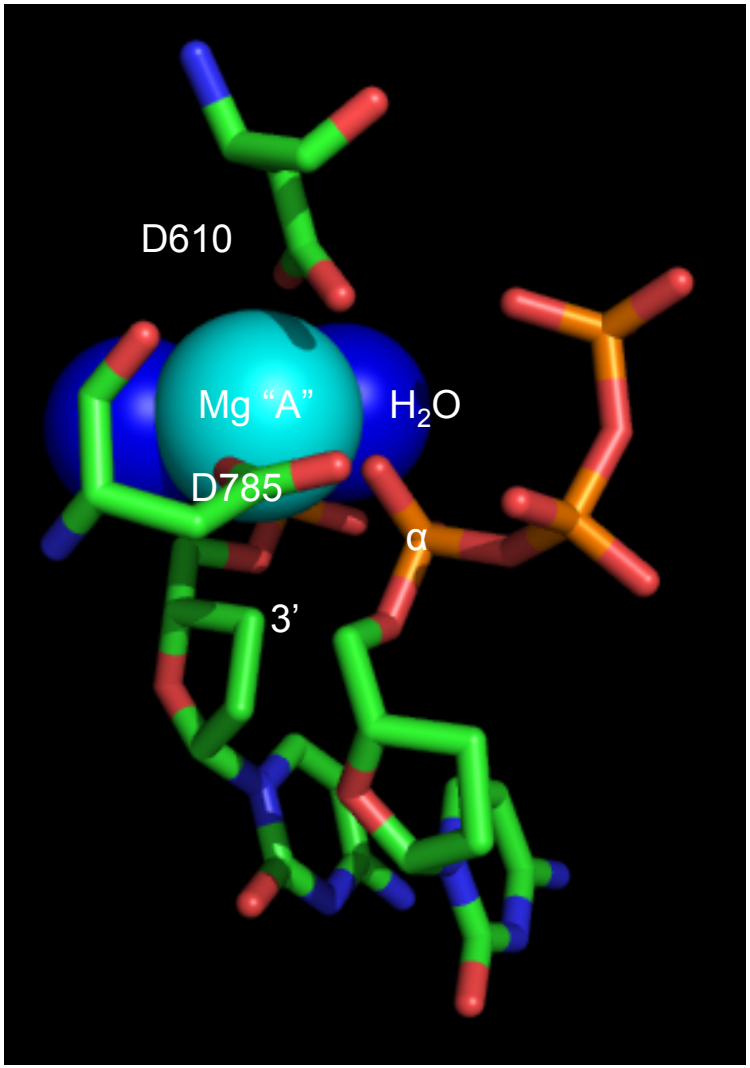
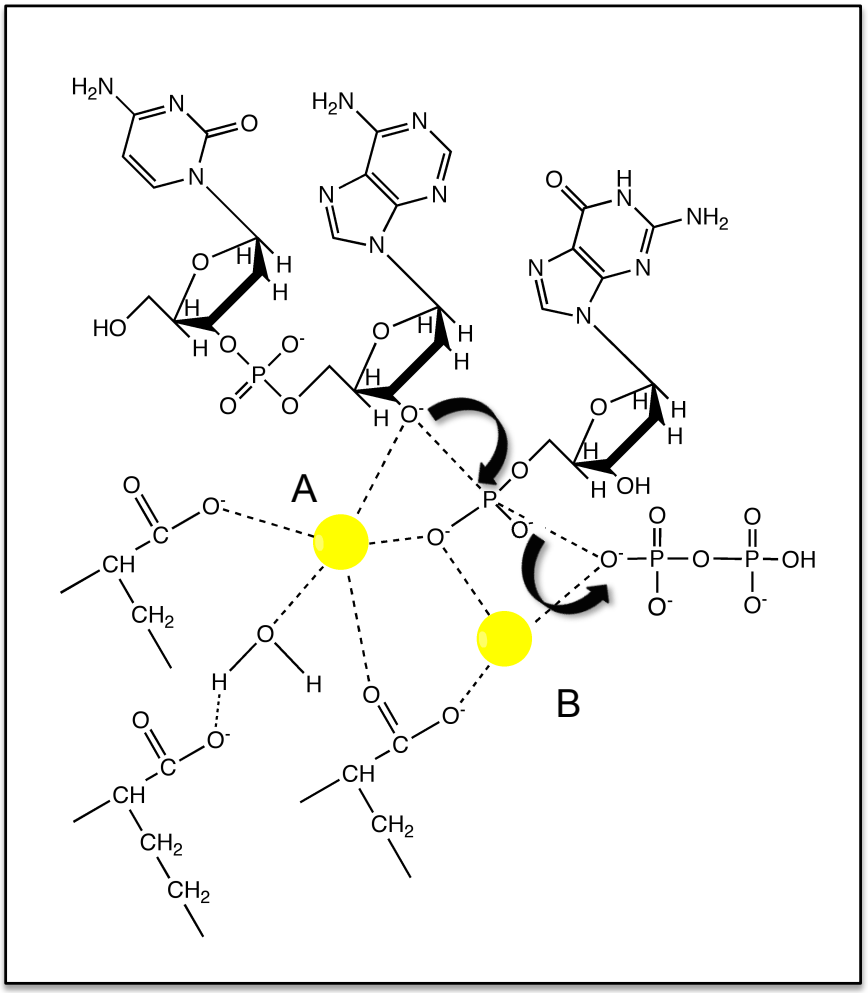




DNA POLYMERASES



Reaction:

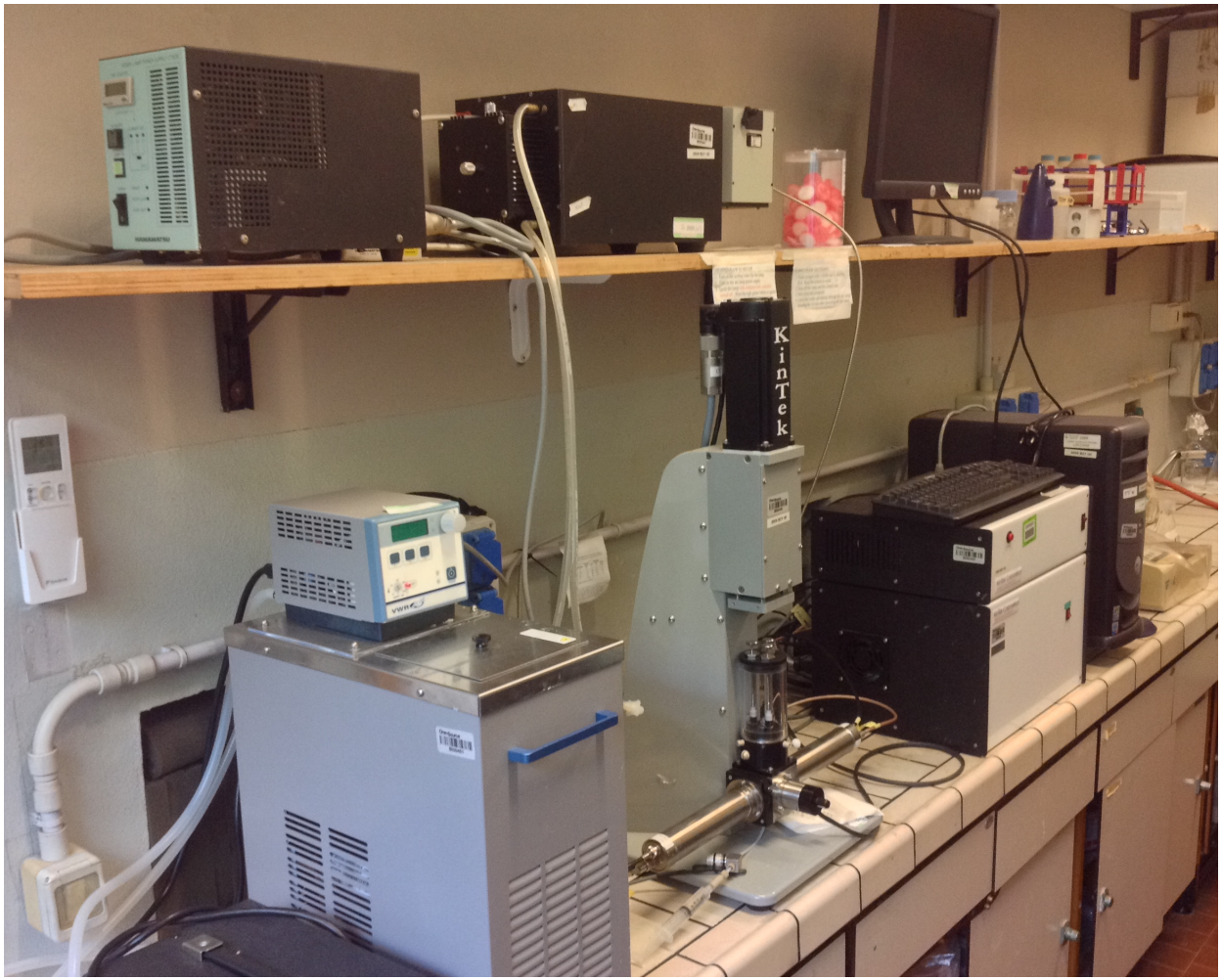
DNA(n) + dNTP DNA(n+1) + PPi + H^+

Reaction intermediate:

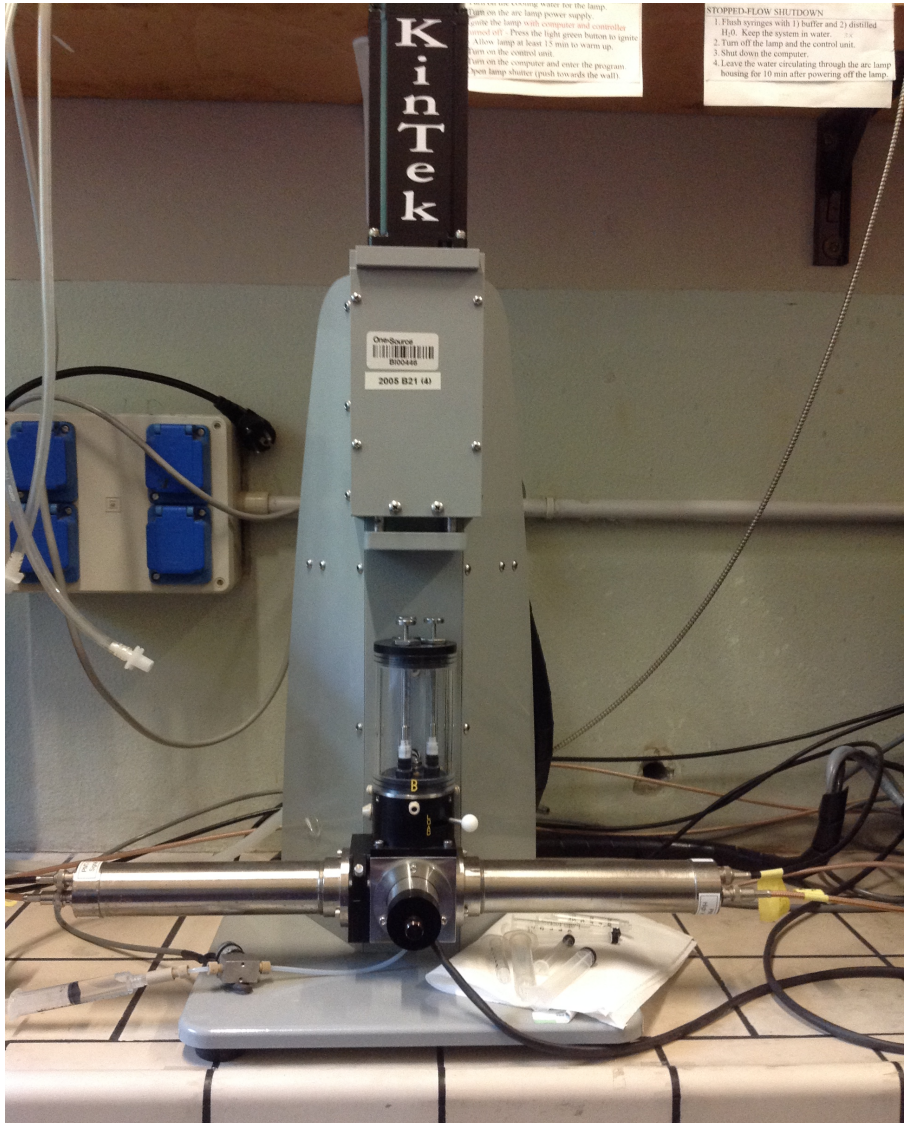
- the nucleophile (3'-O^-) is attacking α-P
- a P-O bond is weakening
- P is penta-coordinated

Correct orientation of the nucleophile is essential for efficient catalysis. The last (n) nucleotide of the primer is paired with the template.
The dNTP is stacked to F667: does this residue play a role in substrate positioning?

DNA POLYMERASES



DNA POLYMERASES



DNA POLYMERASES

Kuchta, Mizrahi, Benkovic, Johnson, Benkovic, 1987, Biochemistry, 26: 8410-8417

Kinetic mechanism of DNA Polymerase I (Klenow)

$$E + DNA_n \underset{k2}{\overset{k1}{\leftrightarrow}} E \bullet DNA_n \underset{k4}{\overset{k3}{\leftrightarrow}} E \bullet DNA_n \bullet dATP \underset{k6}{\overset{k5}{\leftrightarrow}} E^* \bullet DNA_n \bullet dATP \underset{k8}{\overset{k7}{\leftrightarrow}} E^* \bullet DNA_{n+1} \bullet PPi$$

$$\underset{k10}{\overset{k9}{\leftrightarrow}} E \bullet DNA_{n+1} + PPi \underset{k12}{\overset{k11}{\leftrightarrow}} E + DNA_{n+1}$$

dsDNA substrate:

5'TCGCAGCGGTCCA3'
3'AGCGTCGGCAGGTTCCCAAA5'

Elongation:

$$DNA_n + dNTP \Longrightarrow DNA_{n+1} + PPi$$

Pyrophosphorolysis:

$$PPi + DNA_{n+1} \Longrightarrow dNTP + DNA_n$$

DNA POLYMERASES

$$E + DNA_n \underset{k_2}{\overset{k_1}{\leftrightarrow}} E \bullet DNA_n \underset{k_4}{\overset{k_3}{\leftrightarrow}} E \bullet DNA_n \bullet dATP \underset{k_6}{\overset{k_5}{\leftrightarrow}} E^* \bullet DNA_n \bullet dATP \underset{k_8}{\overset{k_7}{\leftrightarrow}} E^* \bullet DNA_{n+1} \bullet PPi$$

$$\underset{k_{10}}{\overset{k_9}{\leftrightarrow}} E \bullet DNA_{n+1} + PPi \underset{k_{12}}{\overset{k_{11}}{\leftrightarrow}} E + DNA_{n+1}$$

Assays of pre-steady-state DNA elongation as a function of [dATP]:

Assays conditions:

- 50 nM Klenow enzyme
- 150 nM DNA
- 2-20 µM dNTP*
- 6.25 mM $MgCl_2$

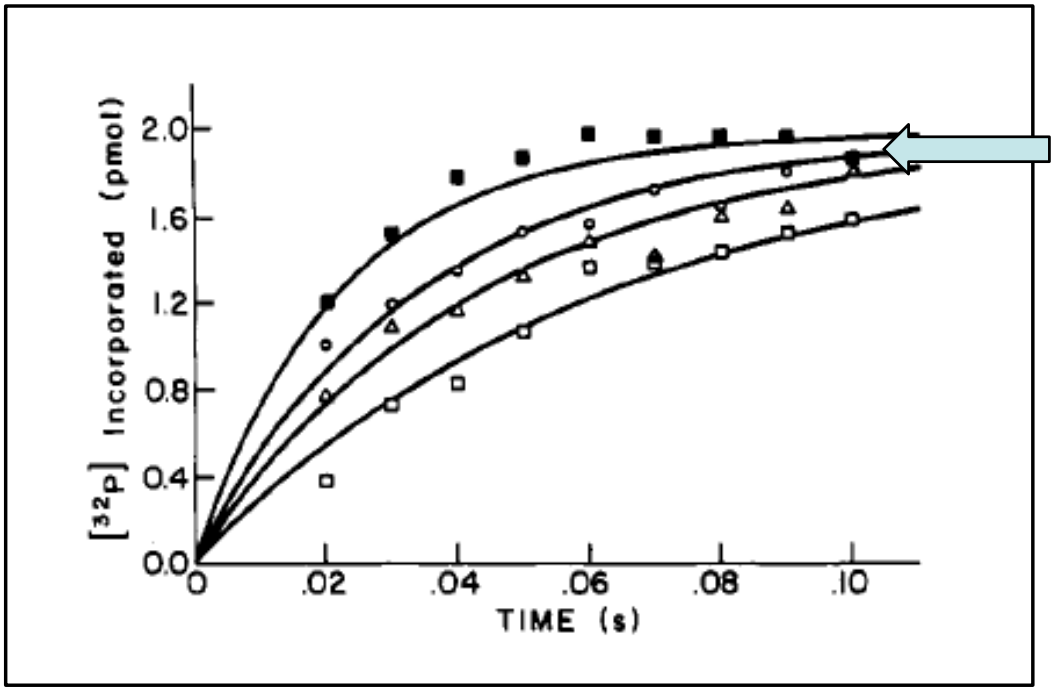
dsDNA substrate (13mer):

5'TCGCAGCGGTCCA3'
3'AGCGTCGGCAGGTTCCCAAA5'

$$E + DNA_n \underset{k_r}{\overset{k_f}{\leftrightarrow}} E \bullet DNA_n \xrightarrow{k_{obs}} E^* \bullet DNA_{n+1} \underset{k_r}{\overset{k_f}{\leftrightarrow}} E + DNA_{n+1}$$

DNA POLYMERASES

$$E + DNA_n \underset{k_r}{\overset{k_f}{\leftrightarrow}} E \bullet DNA_n \xrightarrow{k_{obs}} E^* \bullet DNA_{n+1} \underset{k_r}{\overset{k_f}{\leftrightarrow}} E + DNA_{n+1}$$



3.3, 5, 10, 20 µM dATP*:
(empty squares, triangles, circles, filled squares)

$k_f = 1.2 \bullet 10^7\ M^{-1}s^{-1}$
$k_r = 0.06\ s^{-1}$

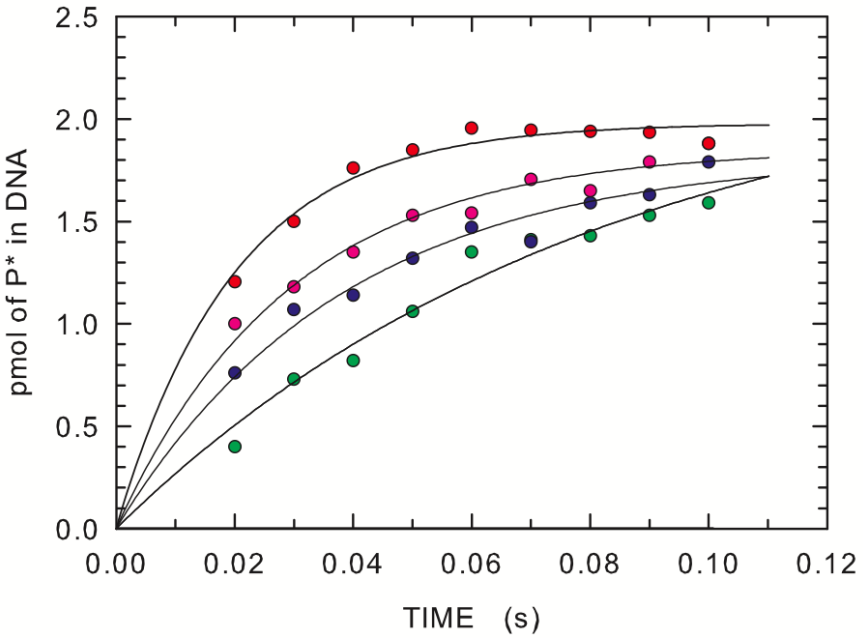
Steady state assays:

- 2 nM Klenow enzyme
- 200 nM DNA
- 1-5 µM dNTP, 5 mM $MgCl_2$

$K_D\ (DNA) = k_r/k_f = 5\ nM$

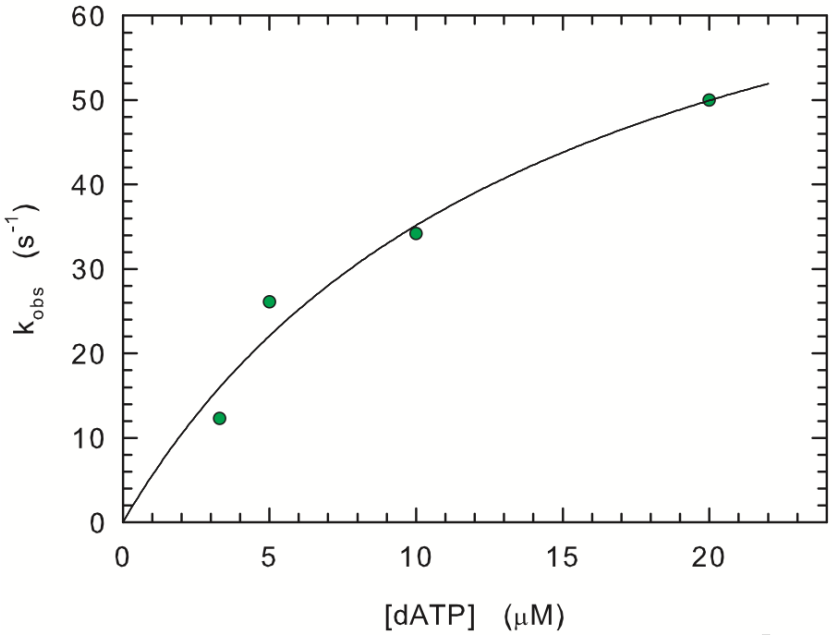
$k_{obs} = 0.06\ s^{-1}$

DNA POLYMERASES



[Product] = 25 nM max [Enzyme] = 50 nM
Less than 1 turnover
[dATP] controls reaction velocity
Determination of kobs
Fitting of the data to a first-order equation
Pseudo-first-order: [dATP]>>[E•DNA]

k_{obs} as a function of [dATP]:



$k_{cat} \cong 50\ s^{-1}$
$K_D \cong 5.5\ \mu M$

DNA POLYMERASES

$$E + DNA_n \underset{0.06\ s^{-1}}{\overset{10^7\ M^{-1}s^{-1}}{\leftrightarrow}} E \bullet DNA_n \underset{k_4}{\overset{K_D\ 5\ \mu M}{\leftrightarrow}} E \bullet DNA_n \bullet dATP \underset{k_6}{\overset{50\ s^{-1}}{\leftrightarrow}} E^* \bullet DNA_n \bullet dATP \underset{k_8}{\overset{k_7}{\leftrightarrow}} E^* \bullet DNA_{n+1} \bullet PPi$$

$$\underset{k_{10}}{\overset{k_9}{\leftrightarrow}} E \bullet DNA_{n+1} + PPi \underset{10^7\ M^{-1}s^{-1}}{\overset{0.06\ s^{-1}}{\leftrightarrow}} E + DNA_{n+1}$$

Assays of pre-steady-state pyrophosphorolysis as a function of [PPi]:

Assays conditions:

- 400 nM Klenow enzyme
- 50 nM DNA*
- 35-210 µM PPi
- 6.25 mM $MgCl_2$

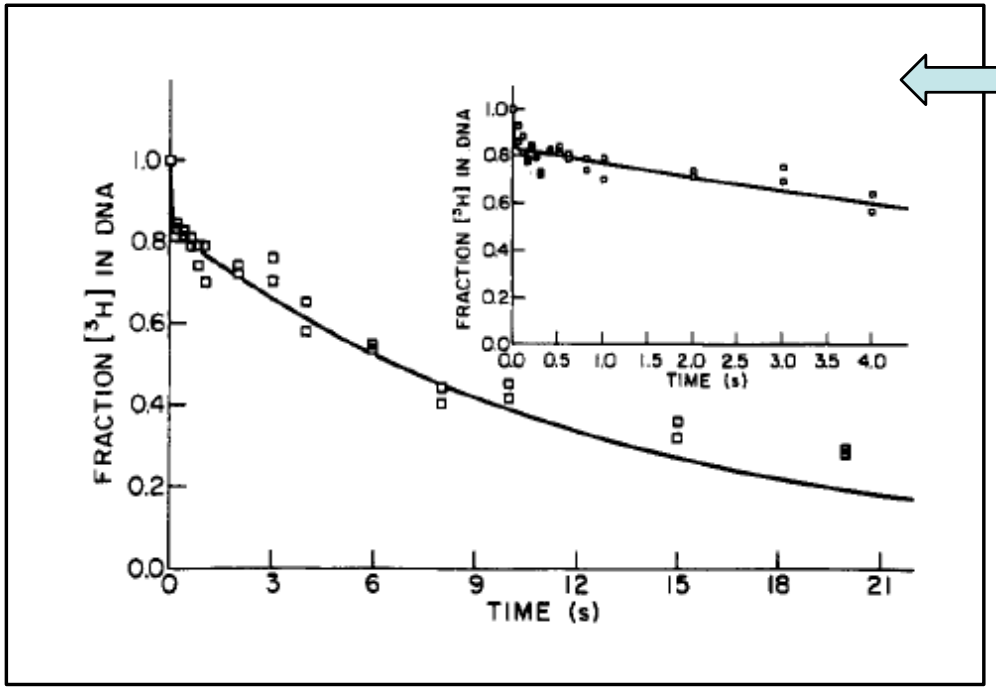
dsDNA substrate (14mer):

5'TCGCAGCGGTCCAA3'
3'AGCGTCGGCAGGTTCCCAAA5'

$$E \bullet DNA_{n+1} + PPi \underset{k_r}{\overset{k_f}{\leftrightarrow}} E \bullet DNA_{n+1} \bullet PPi \xrightarrow{k_{obs}} E \bullet DNA_n + dATP$$

DNA POLYMERASES

The observed kinetics is composed of two phases



140 µM PPi:

k_{obs} (fast phase) = 15 s^{-1}
k_{obs} (slow phase) = 0.1 s^{-1}

Lowering [PPi], the k_{obs} of the slow phase decreases.
Reciprocal plot (rate vs. [PPi]):

- K_m (PPi) = 35 µM
- k_{cat} = 0.12 s^{-1}

DNA POLYMERASES

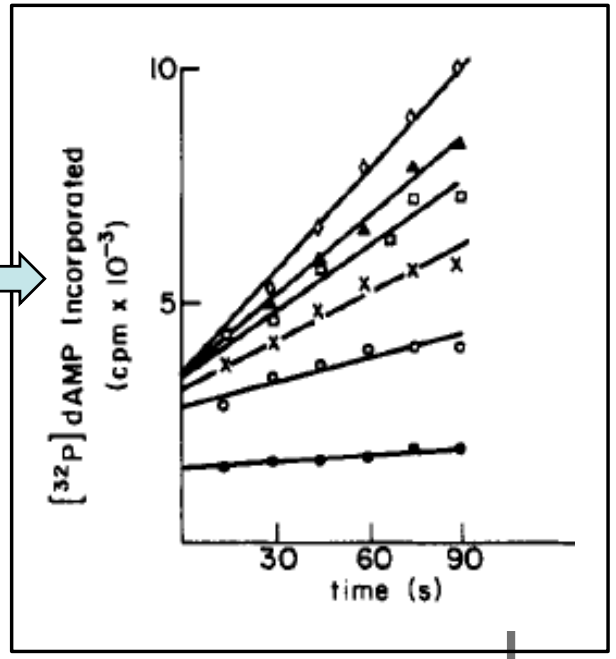
$$E + DNA_n \underset{k_r}{\overset{k_f}{\leftrightarrow}} E \bullet DNA_n \xrightarrow{k_{obs}} E^* \bullet DNA_{n+1} \underset{k_r}{\overset{k_f}{\leftrightarrow}} E + DNA_{n+1}$$

Binding of DNA: $E + DNA_n \underset{k_r}{\overset{k_f}{\leftrightarrow}} E \bullet DNA_n$

Assays conditions:

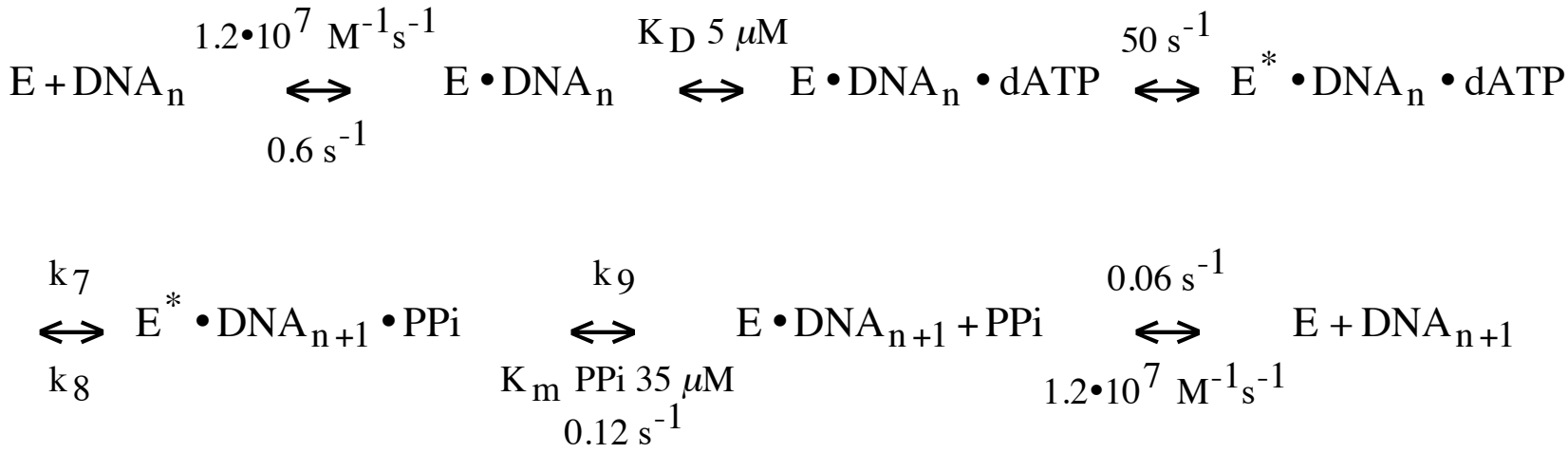
- 5 nM Klenow enzyme
- 2.5-20 nM DNA
- 0.9 µM dNTP*
- 5 mM $MgCl_2$

K_D = 5 nM



- Reaction started with dATP*
- Extrapolation to t_0 of the $[DNA_{n+1}]$
- $[DNA_{n+1}]$ at t_0 as a fucntion of $[DNA_n]$
- Scatchard plot and determination of K_D

DNA POLYMERASES



Determination of K_D for dATP:

- 5 nM Klenow enzyme
- 2 nM DNA (13mer)
- 25-150 nM dATP*
- 5 mM $MgCl_2$
- 60 nM DNA (9mer, trap)

Assay conditions:

- DNA 13mer is added first (30 s incubation)
- simultaneous addition of dATP* and DNA 9mer
- aliquots removed every 15 s
- analysis of DNA*
- DNA* as a function of time
- burst of DNA* at t_0

DNA POLYMERASES

$$E + DNA_n \underset{0.6\ s^{-1}}{\overset{1.2 \cdot 10^7\ M^{-1}s^{-1}}{\leftrightarrow}} E \cdot DNA_n \overset{K_D\ 5\ \mu M}{\leftrightarrow} E \cdot DNA_n \cdot dATP \overset{50\ s^{-1}}{\leftrightarrow} E^* \cdot DNA_n \cdot dATP$$

$$\overset{k_7}{\underset{k_8}{\leftrightarrow}} E^* \cdot DNA_{n+1} \cdot PPi \overset{k_9}{\underset{K_m\ PPi\ 35\ \mu M;\ 0.12\ s^{-1}}{\leftrightarrow}} E \cdot DNA_{n+1} + PPi \overset{0.06\ s^{-1}}{\underset{1.2 \cdot 10^7\ M^{-1}s^{-1}}{\leftrightarrow}} E + DNA_{n+1}$$

Forward reaction (50 s^{-1}) much faster than pyrophosphorolysis (0.12 s^{-1})

The rate constant for dATP dissociation is >> than the rate constant for DNA extension (*F.R. Bryant, K.A. Johnson, S.J. Benkovic, 1983, Biochemistry, 22:3537*)

$$E \cdot DNA_n \leftrightarrow E \cdot DNA_n \cdot dATP \rightarrow E^* \cdot DNA_{n+1} \cdot PPi \rightarrow E \cdot DNA_{n+1} + PPi \rightarrow E + DNA_{n+1}$$

DNA POLYMERASES

William Wallace Cleland, 1975, Biochemistry, 14: 3220-3224

Partition analysis and the concept of net rate constants as tools in enzyme kinetics

$$E + S \underset{k_2}{\overset{k_1}{\leftrightarrow}} ES \underset{k_4}{\overset{k_3}{\leftrightarrow}} EP \xrightarrow{k_5} P + E$$

Net rate constant: would yield the same flux through a step under irreversible conditions

$$E + S \xrightarrow{k'_1} ES \xrightarrow{k'_3} EP \xrightarrow{k'_5} P + E \qquad E_1 \xrightarrow{k'_1} E_2 \xrightarrow{k'_3} E_3 \xrightarrow{k'_5} E_1 \qquad [E_t] = [E_1] + [E_2] + [E_3]$$

Under steady - state conditions :

$$\frac{d[E_1]}{dt} = 0 \quad k'_1 \bullet [E_1] = k'_5 [E_3] \qquad \frac{d[E_2]}{dt} = 0 \quad k'_3 \bullet [E_2] = k'_1 [E_1] \qquad \frac{d[E_3]}{dt} = 0 \quad k'_5 \bullet [E_3] = k'_3 [E_2]$$

DNA POLYMERASES

$$\frac{d[E_1]}{dt}=0 \quad k'_1 \bullet [E_1]=k'_5[E_3] \qquad \frac{d[E_2]}{dt}=0 \quad k'_3 \bullet [E_2]=k'_1[E_1] \qquad \frac{d[E_3]}{dt}=0 \quad k'_5 \bullet [E_3]=k'_3[E_2]$$

$$[E_1]=\frac{k'_5}{k'_1}\bullet[E_3]=\frac{k'_3}{k'_1}\bullet[E_2] \qquad [E_2]=\frac{k'_1}{k'_3}\bullet[E_1]=\frac{k'_5}{k'_3}\bullet[E_3] \qquad [E_3]=\frac{k'_3}{k'_5}\bullet[E_2]=\frac{k'_1}{k'_5}\bullet[E_1]$$

$$\frac{[E_1]}{[E_t]}=\frac{\frac{k'_3}{k'_1}\bullet[E_2]}{\frac{k'_3}{k'_1}\bullet[E_2]+\frac{k'_1}{k'_3}\bullet[E_1]+\frac{k'_1}{k'_5}\bullet[E_1]}=\frac{\frac{1}{k'_1}}{\frac{1}{k'_1}+\frac{k'_1[E_1]}{k'_3\,k'_3[E_2]}+\frac{k'_1[E_1]}{k'_5\,k'_3[E_2]}}=\frac{\frac{1}{k'_1}}{\frac{1}{k'_1}+\frac{1}{k'_3}+\frac{1}{k'_5}}$$

And :

$$\frac{[E_2]}{[E_t]}=\frac{\frac{1}{k'_3}}{\frac{1}{k'_1}+\frac{1}{k'_3}+\frac{1}{k'_5}} \qquad \frac{[E_3]}{[E_t]}=\frac{\frac{1}{k'_5}}{\frac{1}{k'_1}+\frac{1}{k'_3}+\frac{1}{k'_5}} \qquad v=k'_1\bullet[E_1]=\frac{[E_t]}{\frac{1}{k'_1}+\frac{1}{k'_3}+\frac{1}{k'_5}}$$

DNA POLYMERASES

Relationship between the apparent k'_i and the true k_i rate constants

The "net" k'_i constant is equal to the true k_i rate constant multiplied by the fraction of enzyme "i" which is converted (partitioned) to the forward form, e.g.:

$$E_1 \underset{k_2}{\overset{k_1}{\leftrightarrow}} E_2 \xrightarrow{k'_3} E_3 \rightarrow E_1 \qquad E_1 \xrightarrow{k'_1} E_2 \xrightarrow{k'_3} E_3 \rightarrow E_1$$

$$k'_1 = k_1 \bullet (\text{partition of } E_1)$$

$$\frac{d[E_2]}{dt} = -k'_3[E_2] - k_2[E_2] + k_1[E_1] \qquad \frac{d[E_2]}{dt} = -k'_3[E_2] + k_1[E_1]$$

Under steady - state conditions :

$$[E_2](k'_3 + k_2) = k_1[E_1] \quad [E_2] = \frac{k_1[E_1]}{k'_3 + k_2} \quad \text{and} \quad [E_2]k'_3 = k'_1[E_1] \quad [E_2] = \frac{k'_1[E_1]}{k'_3}$$

DNA POLYMERASES

$$E_1 \underset{k_2}{\overset{k_1}{\leftrightarrow}} E_2 \xrightarrow{k'_3} E_3 \rightarrow E_1 \qquad E_1 \xrightarrow{k'_1} E_2 \xrightarrow{k'_3} E_3 \rightarrow E_1$$

$$[E_2] = \frac{k_1[E_1]}{k'_3 + k_2} \qquad [E_2] = \frac{k'_1[E_1]}{k'_3} \qquad \frac{k'_1}{k'_3} = \frac{k_1}{k'_3 + k_2} \qquad k'_1 = k_1 \bullet \left(\frac{k'_3}{k'_3 + k_2}\right)$$

$$\left(\frac{k'_3}{k'_3 + k_2}\right) = \text{partition of enzyme} \qquad k'_i = k_i \bullet \left(\frac{k'_{i+2}}{k'_{i+2} + k_{i+1}}\right)$$

$$E \bullet DNA_n \underset{k_4}{\overset{k_3}{\leftrightarrow}} E \bullet DNA_n \bullet dATP \xrightarrow{k'_5} E^* \bullet DNA_{n+1} \bullet PPi \xrightarrow{k'_6} E \bullet DNA_{n+1} + PPi \xrightarrow{k'_7} E + DNA_{n+1}$$

$$k'_3 = k_3 \bullet [dATP] \bullet \left(\frac{k'_5}{k_4 + k'_5}\right) = k_f \quad \Longrightarrow \quad k_f\text{: forward “net” rate constant}$$

DNA POLYMERASES

$$[DNA_{n+1}] = [DNA_n] \bullet \left(\frac{k_f}{k_f + k_r} \right) \qquad k_f = k_3 \bullet [dATP] \bullet \left(\frac{k'_5}{k_4 + k'_5} \right)$$

$$E \bullet DNA_n \underset{k_4}{\overset{k_3}{\leftrightarrow}} E \bullet DNA_n \bullet dATP \xrightarrow{k'_5} E^* \bullet DNA_{n+1} \bullet PPi$$

k'_5 is << than k_4 (*F.R. Bryant, K.A. Johnson, S.J. Benkovic, 1983, Biochemistry, 22:3537*)

$$[DNA_{n+1}] = [DNA_n] \bullet \frac{[dATP] \bullet \frac{k_3 k'_5}{k_4}}{[dATP] \bullet \frac{k_3 k'_5}{k_4} + k_r} = [DNA_n] \bullet \frac{[dATP] \bullet \frac{k'_5}{K_D}}{[dATP] \bullet \frac{k'_5}{K_D} + k_r} = [DNA_n] \bullet \frac{[dATP] \bullet k'_5}{[dATP] \bullet k'_5 + K_D k_r}$$

$$\frac{1}{[DNA_{n+1}]} = \frac{1}{[DNA_n]} + \frac{K_D k_r}{[DNA_n][dATP]k'_5}$$

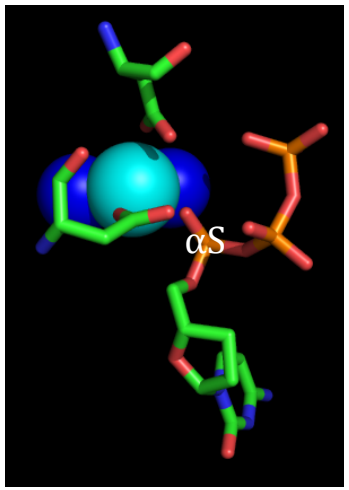
Determination of DNA_{n+1} as a function of [dATP]: the K_D (for dATP) can be estimated. Same method to evaluate the K_D for Ppi.

DNA POLYMERASES

Elongation/pyrophosphorolysis step: equilibrium calculated from [P*] = 2.5

$$E + DNA_n \underset{0.6\ s^{-1}}{\overset{1.2 \cdot 10^7\ M^{-1}s^{-1}}{\leftrightarrow}} E \bullet DNA_n \overset{K_D\ 5\ \mu M}{\leftrightarrow} E \bullet DNA_n \bullet dATP \overset{50\ s^{-1}}{\leftrightarrow} E^* \bullet DNA_n \bullet dATP$$

$$\overset{K_{eq}\ 2.5}{\leftrightarrow} E^* \bullet DNA_{n+1} \bullet PPi \overset{k_9}{\underset{K_D\ PPi\ 100\ \mu M;\ 0.12\ s^{-1}}{\leftrightarrow}} E \bullet DNA_{n+1} + PPi \overset{0.06\ s^{-1}}{\underset{1.2 \cdot 10^7\ M^{-1}s^{-1}}{\leftrightarrow}} E + DNA_{n+1}$$



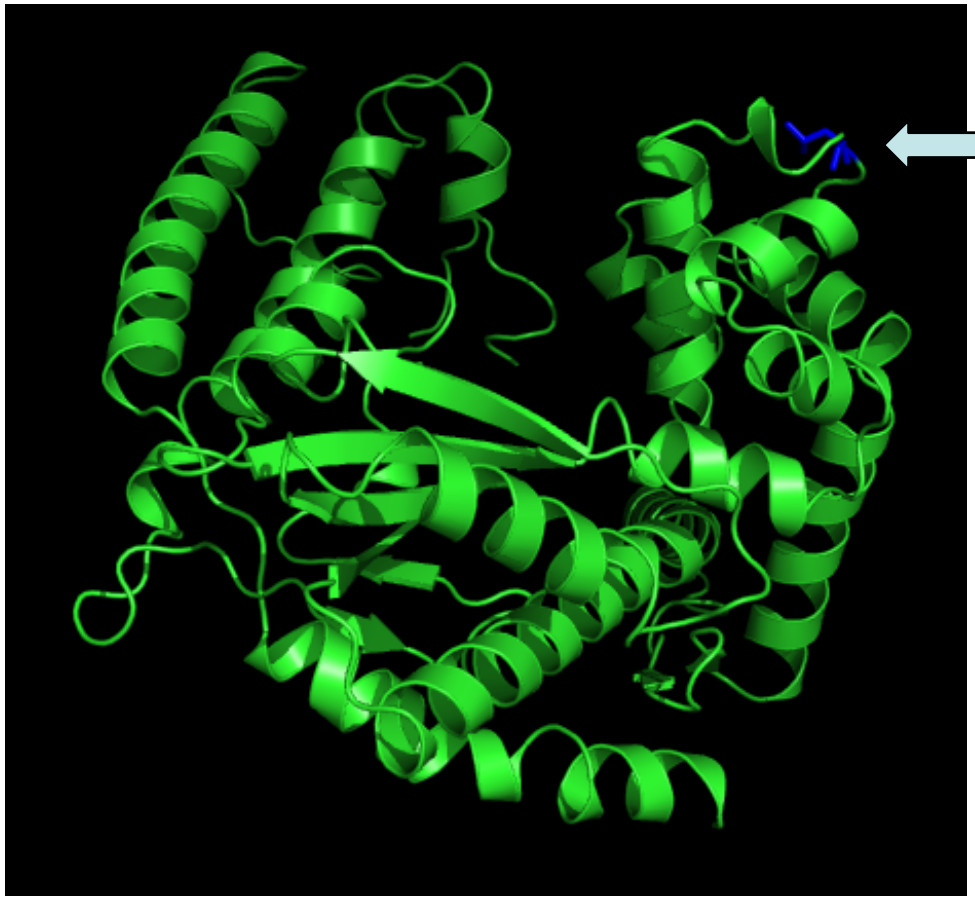
<u>Extension of DNA in the presence of dATPαS</u>:

- 1.6-fold slower under steady state conditions
- pre-steady state rate decreases 3-fold
- first-order rate is 7-folds lower

The pre-chemistry step is rate-limiting. Conformational transition?

DNA POLYMERASES

Pre-chemistry step. *Bermek, Grindley, Joyce, 2011, J. Biol. Chem. 286: 3755-3766*.



L744C
Cysteine modified with fluorophore

dsDNA modified with fluorophore

A A GAGTCAACAGGTC$_H$-3’
A G CTCAG**D**TGTCCAGAGATGG-5’

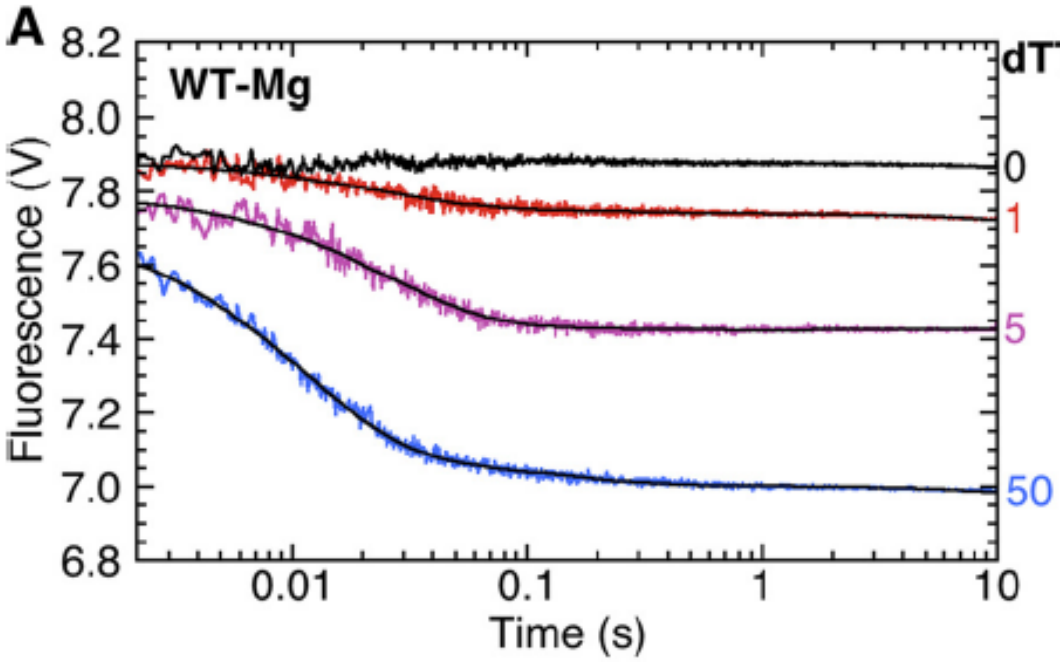
Detection of fluorescence:

excitation 350 nm
emission 400 nm longpass filter

DNA POLYMERASES

Fingers-closure step

The conformational transition triggers the decrease of fluorescence intensity



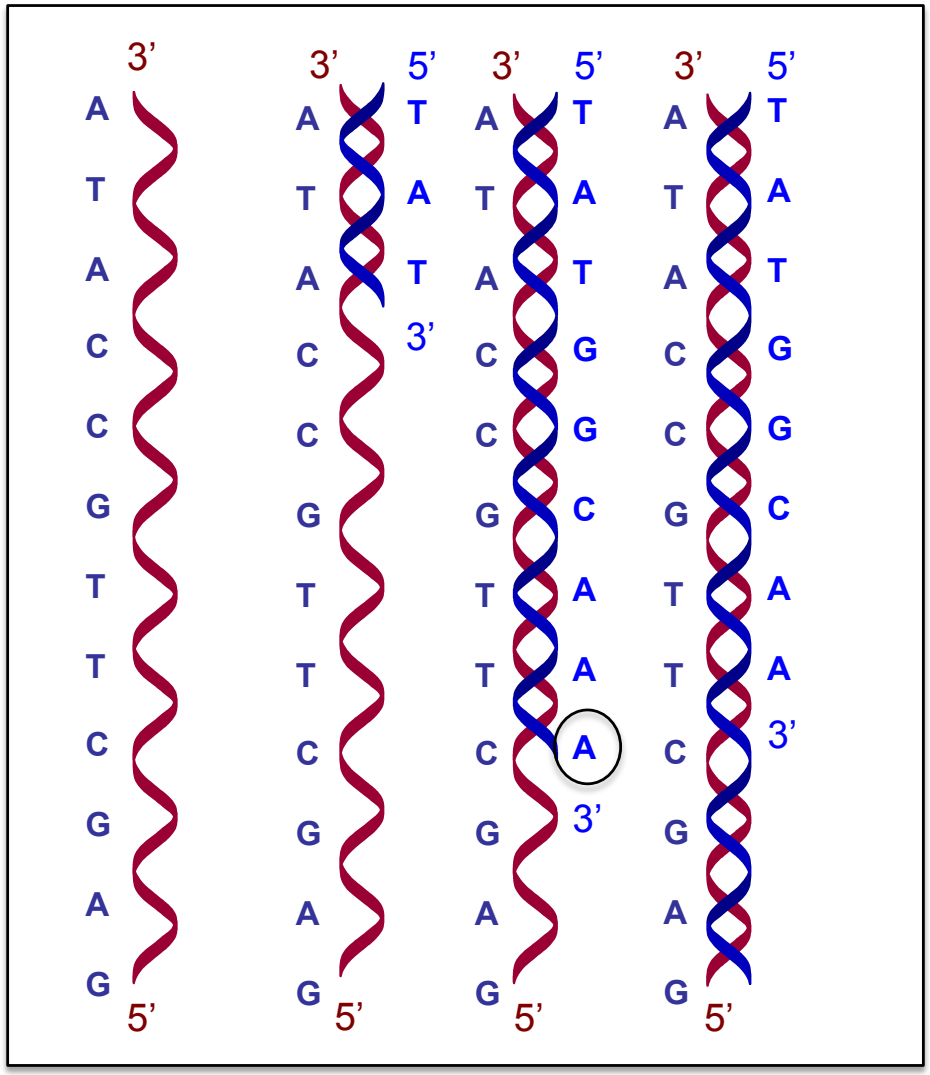
Extension cannot occur (ddC)

Kinetics is biphasic (Mg^{2+}):
- k_{obs} fast phase = 75 ± 18 s^{-1}
- k_{obs} slow phase = 6.3 ± 1.8 s^{-1}

In the presence of Mn^{2+}:
- k_{obs} fast phase = 91 ± 6 s^{-1}
- k_{obs} slow phase = 6.3 ± 2.3 s^{-1}

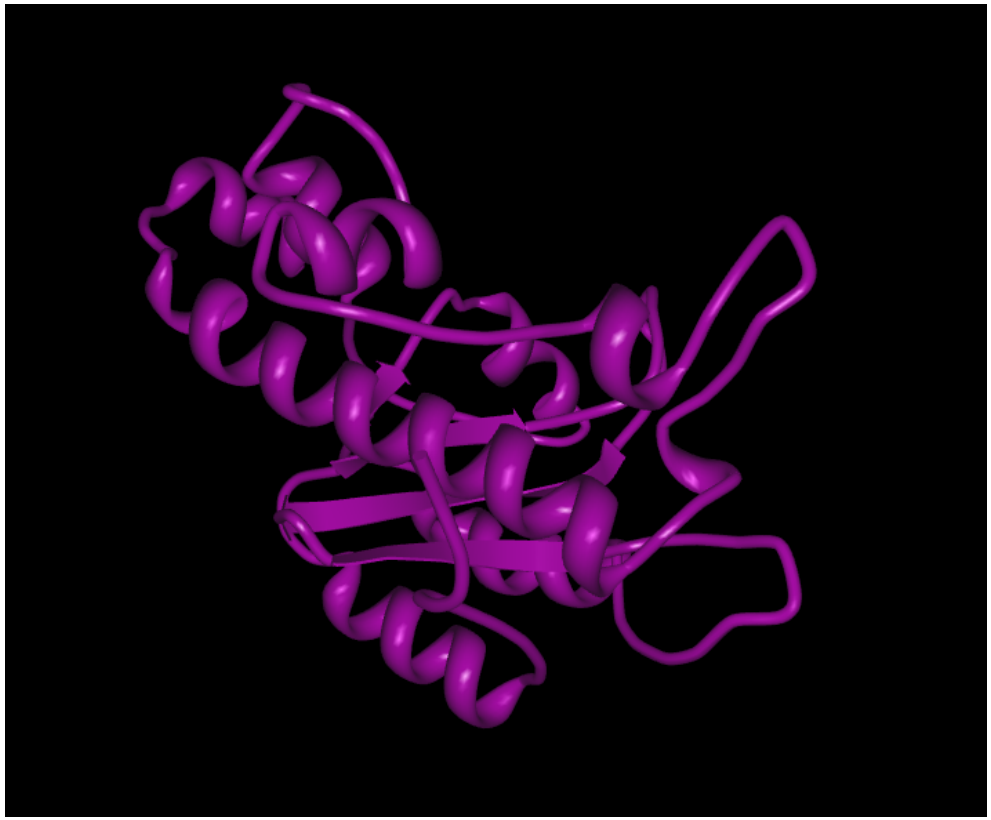
At low [Mg^{2+}] fingers closure takes place: binding of Mg^{2+}-dNTP (metal "B").
Further step: binding of metal "A" (low affinity).

DNA POLYMERASES



DNA POLYMERASES

A domain is responsible for the exonuclease (3'-5') activity of the enzyme (single protein)
A subunit possessing exonuclease activity associates to the polymerase (two proteins)
Exonuclease activity increases up to a factor of 10^4 the fidelity of replication



The exonuclease "proofreads" replication errors. Tasks of exonuclease action (also denoted proofreading action):

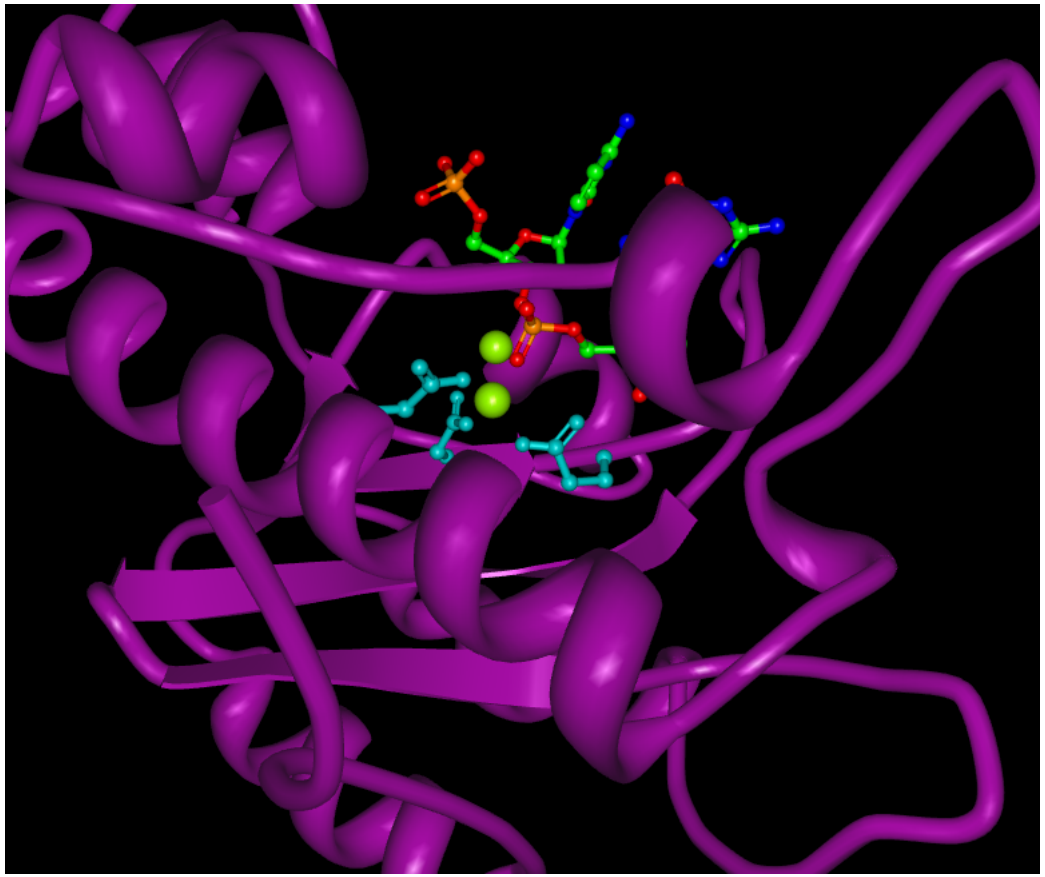
- melting the double-strand containing the mismatch (non Watson-Crick pairing)
- cleaving the phosphodiester bond which corresponds to the mismatch

Polymerase activity:
H^+ is a reaction product

Exonuclease activity:
water is a substrate

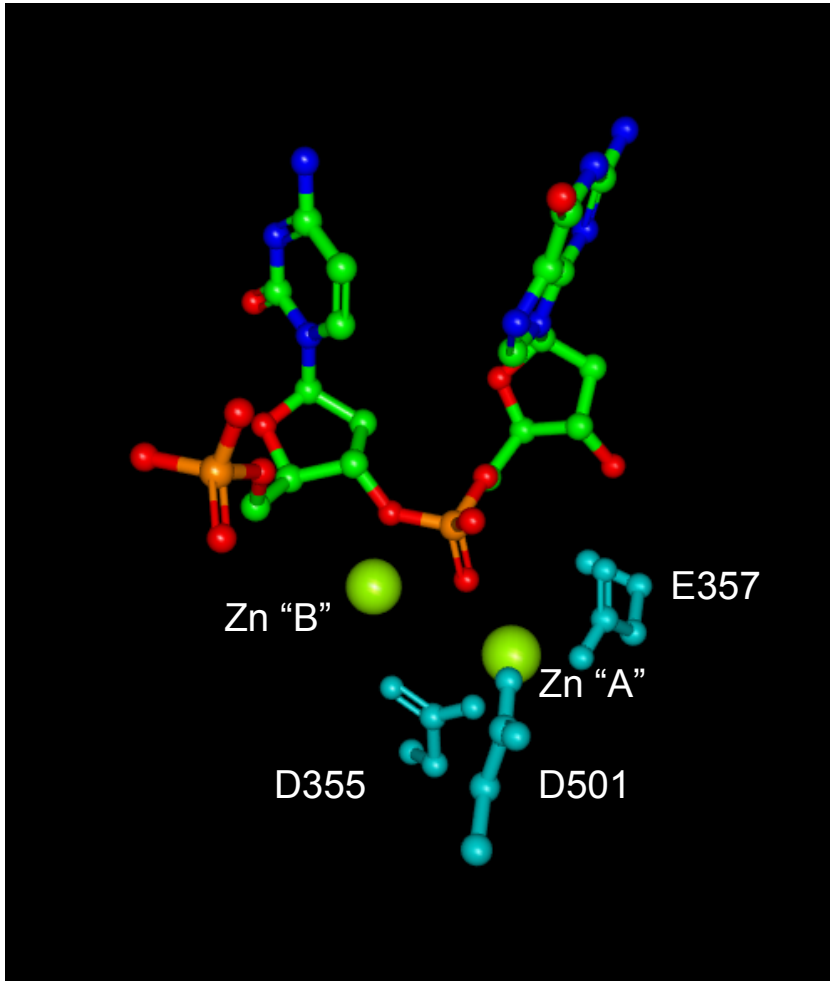
DNA POLYMERASES

Exonuclease domain of *Escherichia coli* DNA Polymerase I



Two metals (yellow) are present in the active site: Mg^{2+}, Mn^{2+}, or Zn^{2+}. Three acidic residues (D355, D501, and E357, cyan) are essential for the exonuclease activity.

DNA POLYMERASES



Metal “B” (octahedral) is coordinated to:

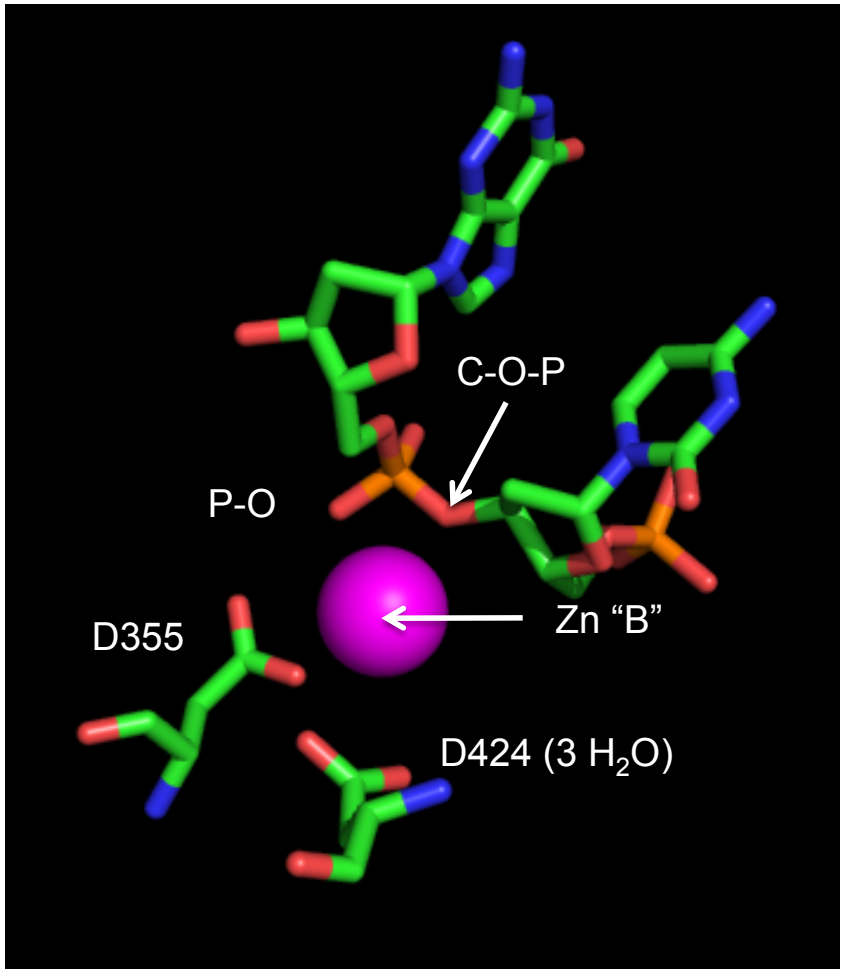
- D355
- 2 P oxygens
- 3 H20

Metal “A” (tetrahedral) is coordinated to:

- D355, D501 and E357
- 1 P oxygen

Both metals are essential for activity.
The pH dependence of activity features a mid-point at about 7. Catalytic group?
Histidines have pKa values at neutral pH.
No Histidines present in the active site.
Catalytic group is presumably Metal “A”.

DNA POLYMERASES



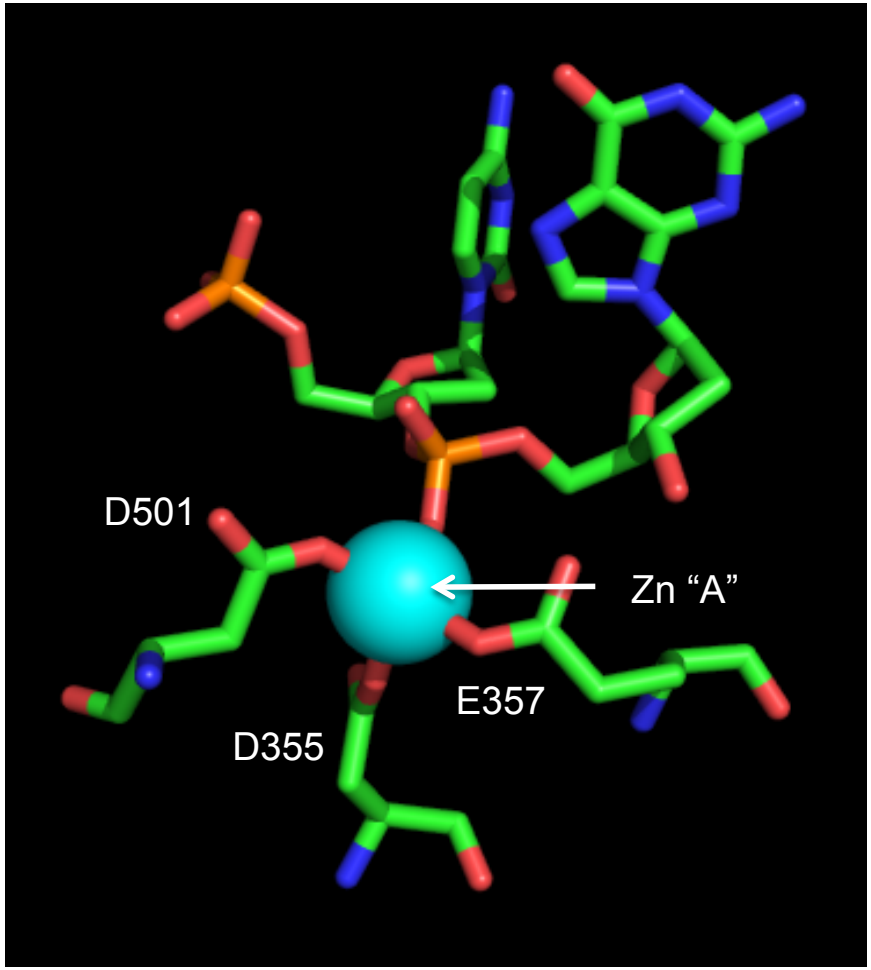
Metal "B" (octahedral) is coordinated to:
- D355
- P oxygen, C-O-P
- 3 H_20

Metal "A" (tetrahedral) is coordinated to:
- D355, D501 and E357
- 1 P oxygen

D424:
- Bridging 3 water molecules
- Coordination to Zn "B"

Catalysis:
- activation of a water molecule
- OH^- as nucleophile

DNA POLYMERASES



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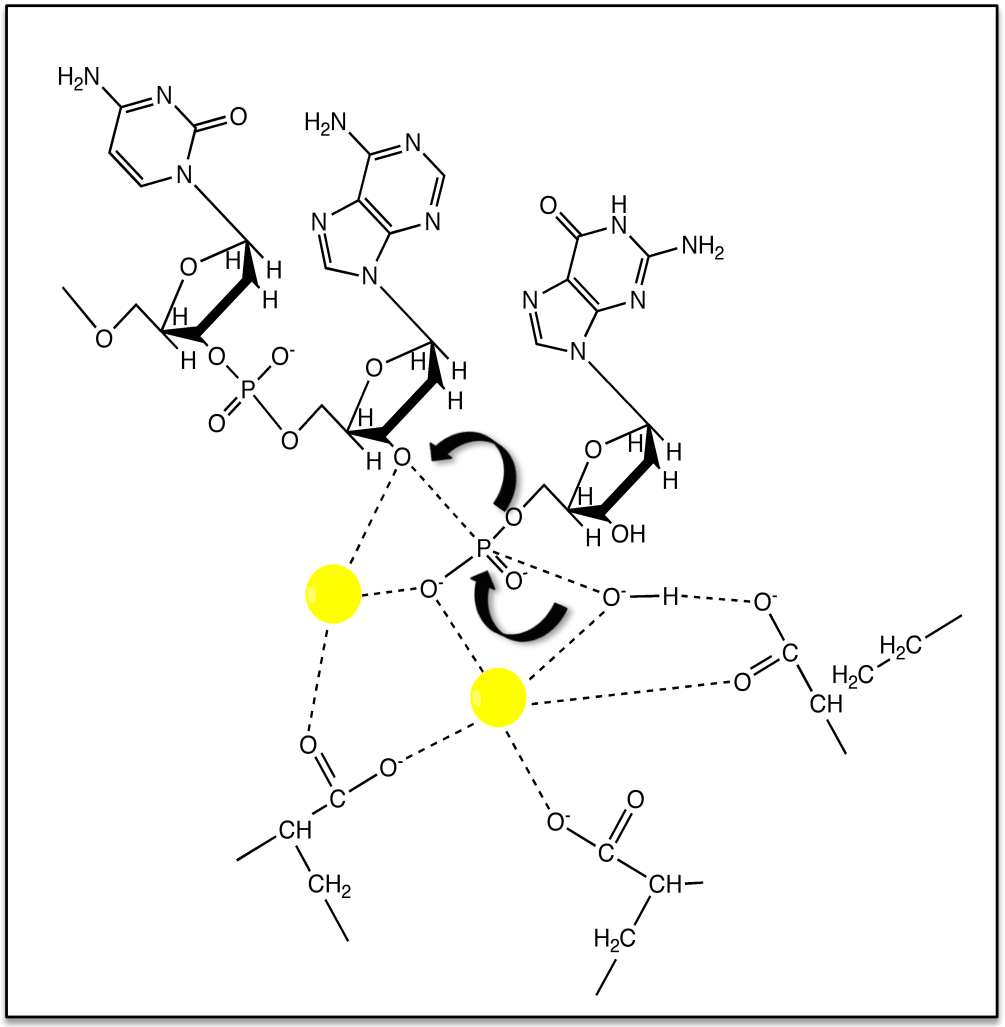
DNA POLYMERASES



Reaction:

DNA(n) + H_2O ➡ DNA(n-1) + dNMP

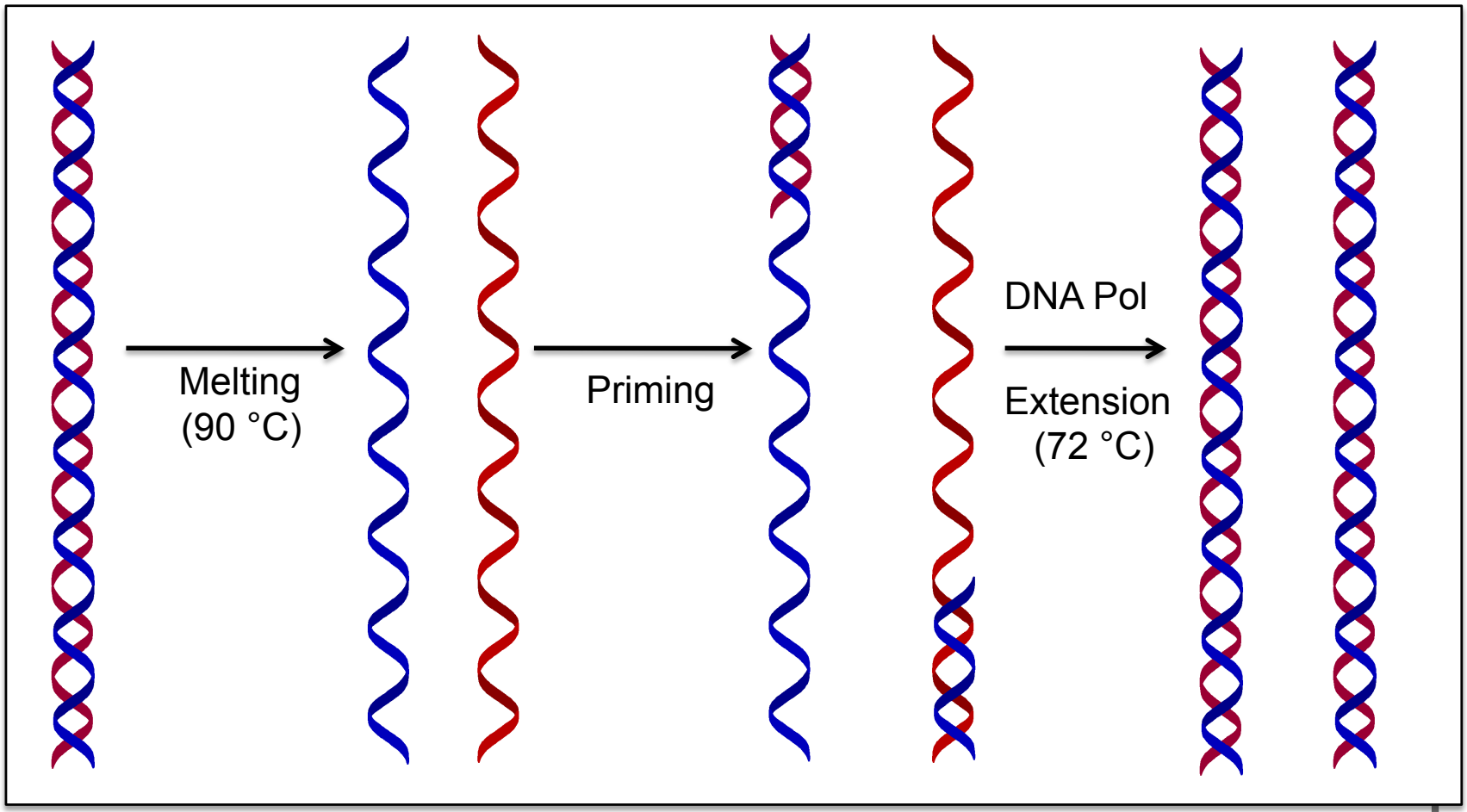
Reaction intermediate:

- the nucleophile (OH^-) is attacking P
- a P-O bond is weakening
- P is penta-coordinated

Correct orientation of the nucleophile is essential for efficient catalysis. The last nucleotide of the primer is not paired with template. Substrate is ssDNA.
The nucleophile is coordinated to Metal "B".
Hoe many phosphodiester bonds wll be hydrolized?

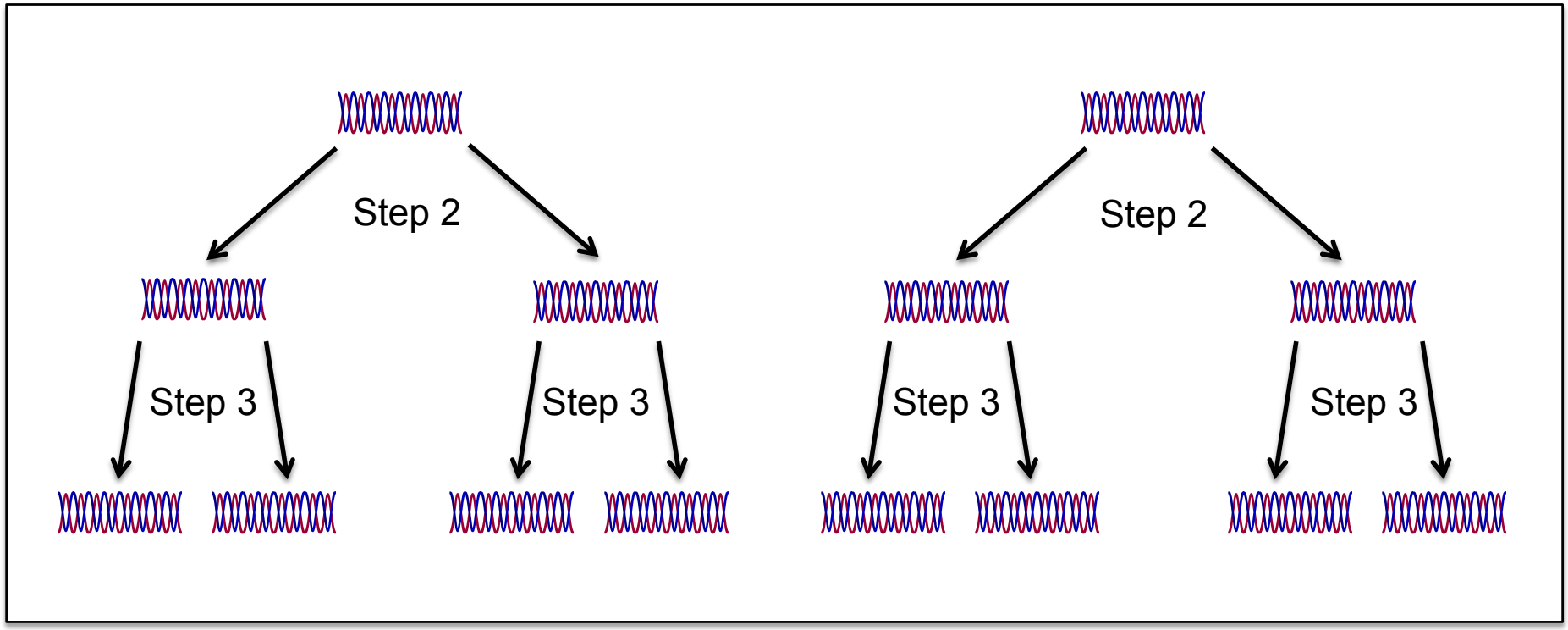
DNA POLYMERASES

Polymerase-Chain-Reaction (PCR): dNTPs, Mg^{2+}, Template DNA, Primers



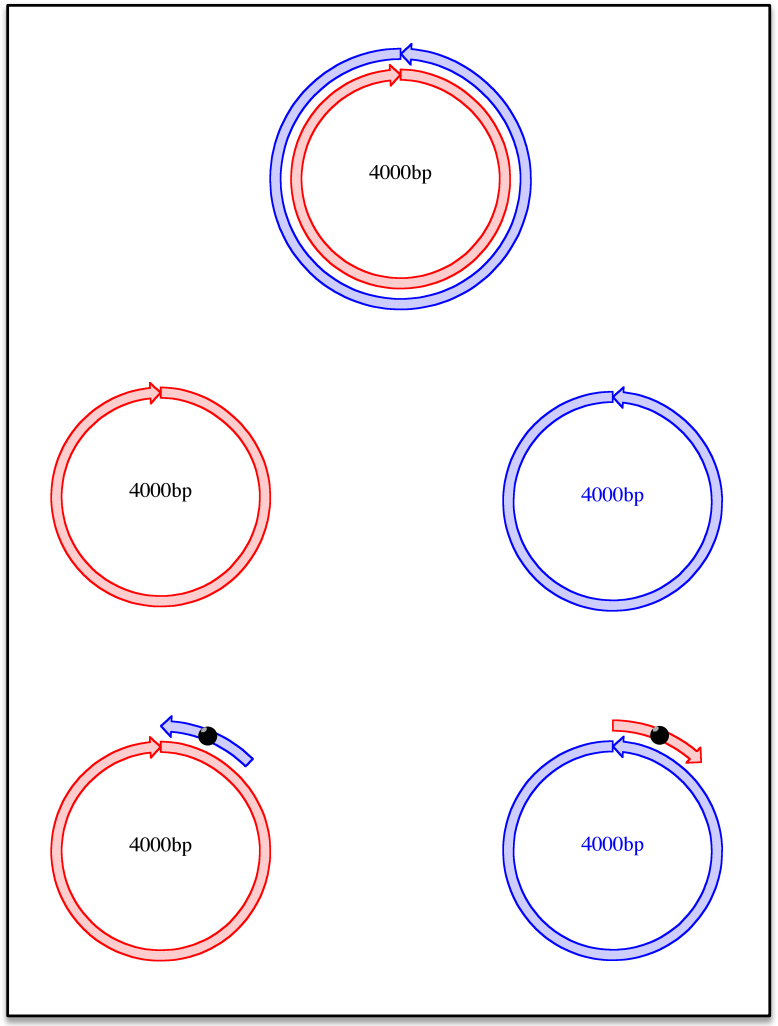
DNA POLYMERASES

Amplification of DNA molecules: repeat the initial cycle for n-times (e.g. 30)



Final yield of DNA molecules: 2^n (n = number of steps).

DNA POLYMERASES



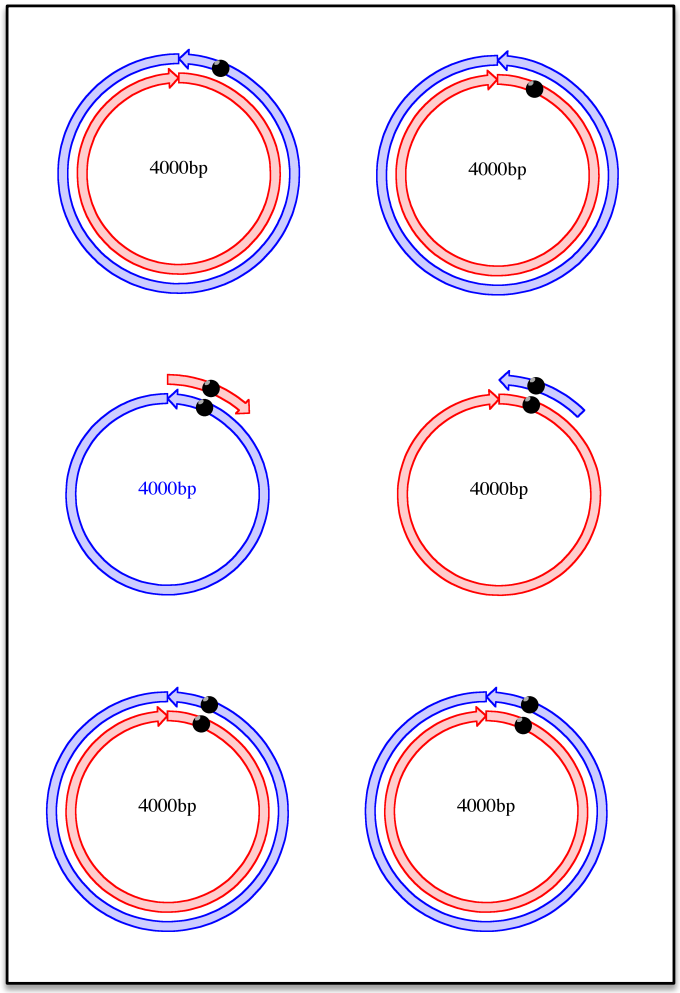
Plasmid mutagenesis

Plasmids are extra-chromosomal genetic elements. They contain non-essential genes. When compared with chromosomes, their molecular mass is usually much lower. They can be replicated in vitro using appropriate primers.

First step: melting (90 °C), two single-stranded (ss) circular DNA molecules are obtained.

Second step: annealing with 2 mutagenic primers (mutation = black circle). Primers in excess over the plasmid.

DNA POLYMERASES



Third step, extension: double-stranded (ds) hybrid is obtained. Primers were added in excess. Repeat the steps 1-3.

Melting, annealing with mutagenic primer.

Extension. Mutant plasmids are enriched over wt and hybrid molecules.