Mechanisms of DNA Synthesis

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Advanced Molecular Biology
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Introduction

Today's topic concerns the fundamental mechanism of DNA replication as exemplified by enzymes from *E. coli*. Nearly identical mechanisms are found in all other polymerases, both prokaryotic and eukaryotic, so only the differences will be mentioned when discussing these other enzymes.

DNA synthetic mechanisms are fundamentally important with respect to the transfer of genetic information from one generation to the next. They are particularly important in maintaining this information. They are also involved in mechanisms for repairing damage to the DNA from external mutagenic agents between rounds of DNA replication.

Discovery of DNA Polymerase

The first DNA synthetic enzymes was discovered by Dr. Arthur Kornberg and his collaborators in the late 50's. They initially found that cell-free extracts from bacteria were able to incorporate ^{3}H-thymidine into DNA much as could the intact cells, but at a very reduced rate.

The first event in these extracts was the conversion of the thymidine into dTTP, and if they used ^{3}H-dTTP the incorporation was much more efficient. Indeed all four nucleotides were being incorporated into DNA very efficiently.

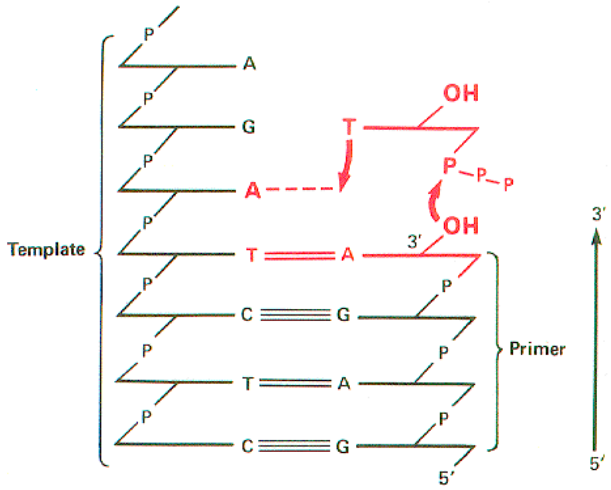
Using this as an assay for *in vitro* DNA synthesis they purified the major DNA synthetic enzyme from the bacterial cells. The reaction that this enzyme, now known as DNA polymerase I, mediates is:

$$\begin{matrix} \text{dATP} \\ \text{dTTP} \\ \text{dGTP} \\ \text{dCTP} \end{matrix} + \text{DNA} \xrightarrow[\text{DNA polymerase}]{Mg^{++}} \text{more DNA} + PP_i \rightarrow 2P_i$$

The requirement for Mg^{++} became apparent during the purification of the enzyme as well as the requirement for DNA in the reaction.

DNA Replication

Two Roles for DNA in the DNA Polymerase Reaction



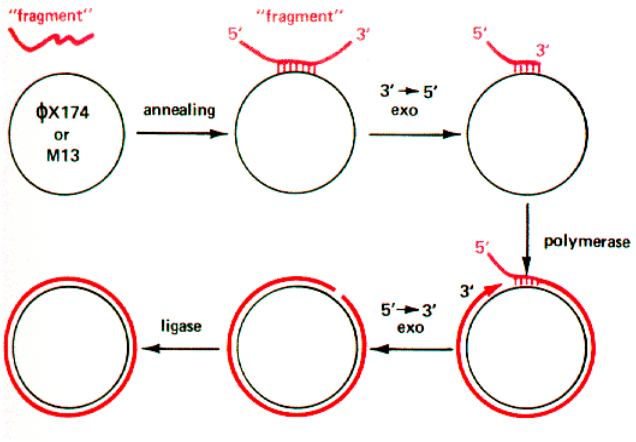
Role of Template

The DNA was found to have two roles in the synthetic reaction. The first was that of a template to direct the order of incorporation of new nucleotides. This was demonstrated several ways.

1. First the base composition of the product was the same as the template DNA.

2. Second, the frequency of dinucleotides was the same.

3. But the most convincing evidence was a series of synthetic steps that replicates the complete genome of ØX174 utilizing the synthetic capacity of DNA polymerase I. This means that all of the genes present within the 5000 base pair genome of ØX174 were replicated accurately.



The Role of DNA as Primer

The second role that DNA serves in the DNA polymerase reaction is that of a primer. All of the newly synthesized DNA is covalently attached to preexisting DNA chains.

1. Proper deoxynucleoside triphosphate is chosen according to the Watson-Crick base pairing rules.

2. The 3' hydroxyl group of the primer strand, known as the primer terminus, attacks the nucleotide splitting off the terminal pyrophosphate group and the enzyme advances one base pair.

3. DNA chains only grow at the 3' end and the direction of synthesis is referred to as 5' to 3' growth. We always signify the 3' end of a DNA chain with an arrowhead.

4. The primer terminus to which the deoxynucleoside triphosphates were added had to be base paired with a single-stranded segment of DNA that was to serve as template.

5. This requirement for a free 3' hydroxyl primer terminus meant that DNA polymerase could not utilize many types of DNA normally found in nature.

RNA Polymerase Can Start Chains

One reason that the requirement for a primer terminus seemed so unusual was that all of these DNA molecules were excellent templates for the enzyme RNA polymerase. RNA polymerase is responsible for transcribing the genetic information from DNA into RNA and this enzyme can initiate RNA chains. All known DNA polymerases require a primer terminus.

Another major difficulty with this DNA synthetic mechanism is that the chains only grow in the 5' to 3' direction. No DNA polymerase has been found that can extend the 5' ends of DNA chains. This causes a problem with respect to the replication of the two daughter strands at a replication fork which of course, have opposite polarities.

Nearest Neighbor Dinucleotide Analysis

An understanding of this chemical mechanism and its unidirectional mode of DNA synthesis allowed a chemical demonstration that the two chains of DNA were antiparallel.

If we assume that the chains were parallel, then we would expect the frequency of the dinucleotide A_pG to be identical to the frequency of the dinucleotide T_pC.

If, on the other hand, the chains were antiparallel, then we would expect the frequency of A_pG to be equal to the frequency of C_pT and not necessarily related to the frequency of T_pC:

```
5'¾¾¾¾→  3'                    5'¾¾¾¾→  3'
    ApG                            ApG
    TpC                            TpC
5'¾¾¾¾→  3'                    3'←¾¾¾¾  5'

PARALLEL    AG = TC        ANTIPARALLEL    AG = CT
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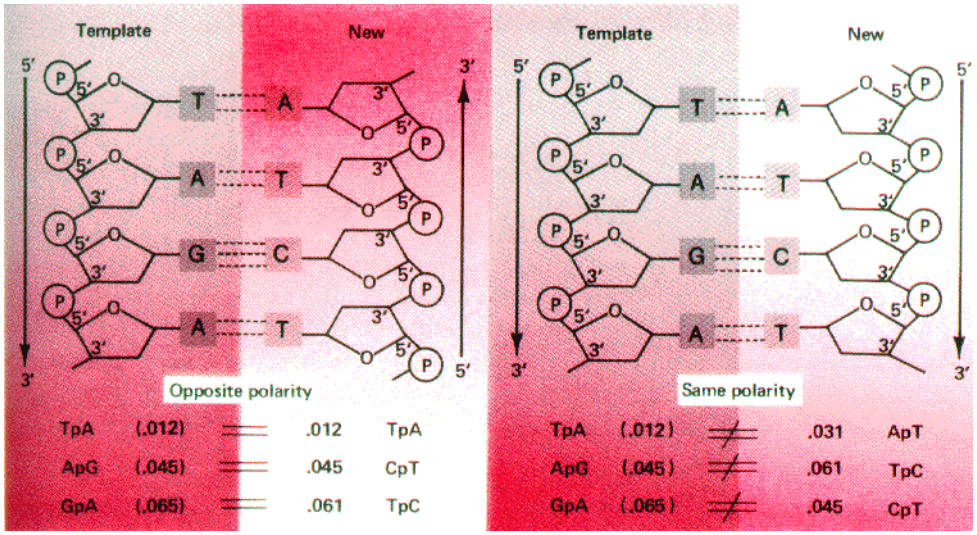
The frequencies of dinucleotides in nearest neighbor experiments is always equal to the frequency of the complementary dinucleotide read in the opposite direction. This is not true for the complementary sequence read in the same direction, hence the chains of duplex DNA must be anti-parallel.

DNA Replication



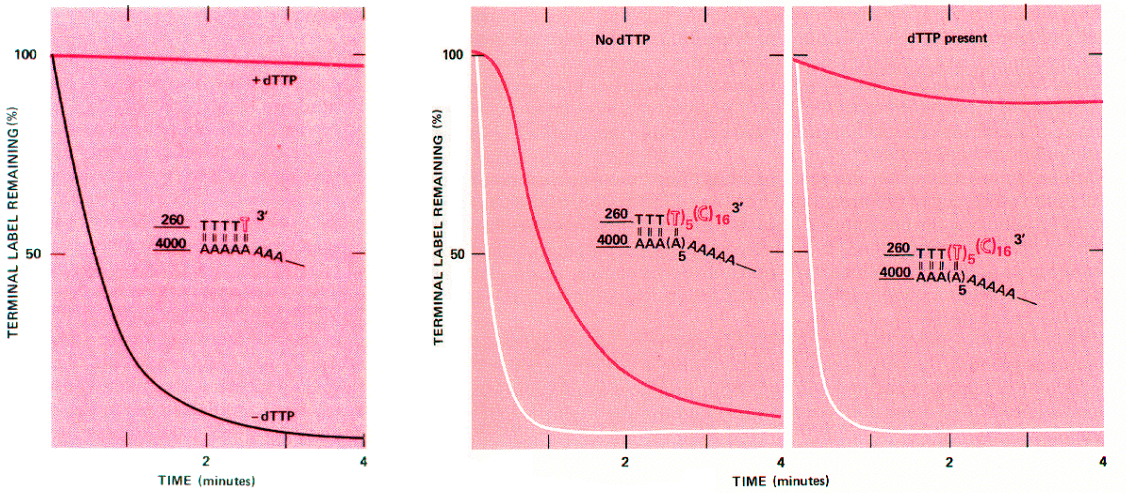
Exonucleases Associated with DNA Polymerase

Proofreading 3' Exonuclease

The purified DNA polymerase contains two exonuclease activities. The enzyme contained both a 3' exonuclease activity, which hydrolyzes DNA chains beginning at the 3' termini and degraded the DNA hydrolytically to mononucleotides.

The enzyme also possesses a 5'-exonuclease which was capable of hydrolytically degrading DNA near the 5' end of DNA. This activity released short oligonucleotides 2 to 10 bases in length as well as mononucleotides from the 5' end of DNA chains.

The presence of the 3'-exonuclease was curious since the synthetic activity of DNA polymerase used the 3' terminus as a primer.



It was found that this nuclease would degrade a primer terminus only if the 3'-terminal nucleotide was not base paired with the template. Under conditions of DNA synthesis the enzyme extended a base paired 3' terminus, but could not extend

a mismatched primer terminus, instead it would degrade it and only begin extending the 3' end when it was base paired with a template.

These data suggest that the function of the 3' exonuclease is to proofread the DNA synthetic process and if an incorrect nucleotide has been incorporated, those mismatches will be detected and removed before further chain elongation occurs.

DNA synthesis is very faithful because every nucleotide is checked twice. It is first selected by the Watson-Crick base pairing rules prior to incorporation into the DNA chain, and it is checked again by the 3' exonuclease prior to being extended.

There is genetic evidence that such an error-correcting exonuclease does indeed fill this role *in vivo*.

Strains of T4 with higher than the spontaneous frequencies of mutation are referred to as mutator strains. In addition, mutants were found which had a lower than normal level of spontaneous mutation and were referred to as anti-mutators.

DNA polymerases induced by both mutator and anti-mutator strains were isolated and the relative rates of exonuclease and polymerase activities in the isolated enzymes was compared. The mutator strains had a markedly reduced level of 3' exonuclease relative to DNA polymerase activity. On the other hand, the anti-mutator strains had a much more active 3' exonuclease relative to polymerase.

These data show a direct correlation of the amount of 3'-exonuclease activity with increased fidelity of replication.

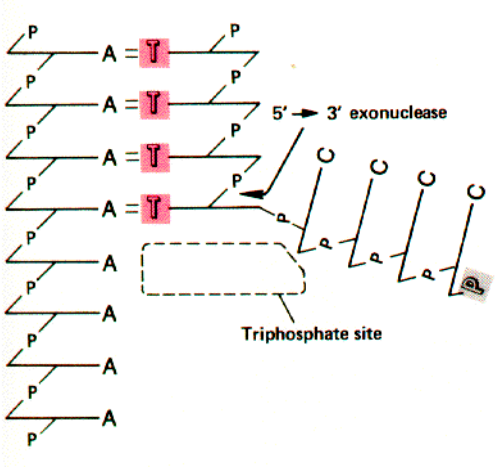
It also shows that the rate of spontaneous mutation is not determined by a random process. Therefore the spontaneous mutation frequency is a genetically variable and selectable characteristic.

The presence of the 3' exonuclease appears to be a common property of all known bacterial DNA polymerases. Some eukaryotic enzymes have been reported to be missing such a proofreading function and these same enzymes have been demonstrated to have a very high frequency of misincorporation *in vitro*.

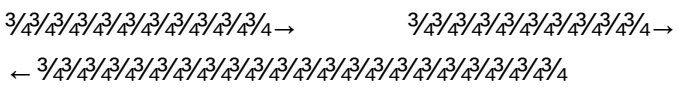
Recent work in Bob Lehman's lab has shown that the major DNA polymerase isolated from *Drosophila* has an intrinsic 3' exonuclease but it is masked by the presence of a 74 kilodalton subunit. Removal of that subunit generates a functional DNA polymerase that displays an active 3' exonuclease.

Role of the 5' Exonuclease

The role of the 5' exonuclease associated with DNA polymerase I also became apparent once its mechanism and specificity were determined. The 5' exonuclease attacks 5' ends of DNA and allows the enzyme to work at nicks in a DNA chain:



3' 5'



This molecule has several primer termini but it has no single-stranded template.

The 5' exonuclease allows simultaneous degradation of the 5' end at a nick and extension of the 3' end by the synthetic activity.

This seemingly futile reaction is referred to as nick translation since the nick is effectively moved along the DNA molecule no net DNA is made.

However, this reaction is good for catalyzing repair of damage to DNA. Clearly if there were damaged bases in the DNA annealed to the template, this damage would be degraded and replaced with new nucleotides.

Role of Nick Translation in Repair of DNA

One major form of damage to DNA, caused by UV light, is the dimerization of adjacent pyrimidines, most frequently, thymine dimers. UV cross-links two adjacent double bonds to form a cyclobutane ring. If this DNA is irradiated prior to nick-translation, then it was shown that the DNA polymerase could excise the thymine dimers and replace them with normal thymines.

The observation that DNA polymerase I can hydrolyze and remove damaged nucleotides from DNA gave a molecular basis for the pathway of repair known as excision repair.

In order for damaged bases to removed from DNA by the nick translation mechanism their clearly has to be a way to specifically introduce nicks near the site of damage.

In the excision repair pathway, recognition of damaged bases results directly in the incision of the phosphodiester backbone.

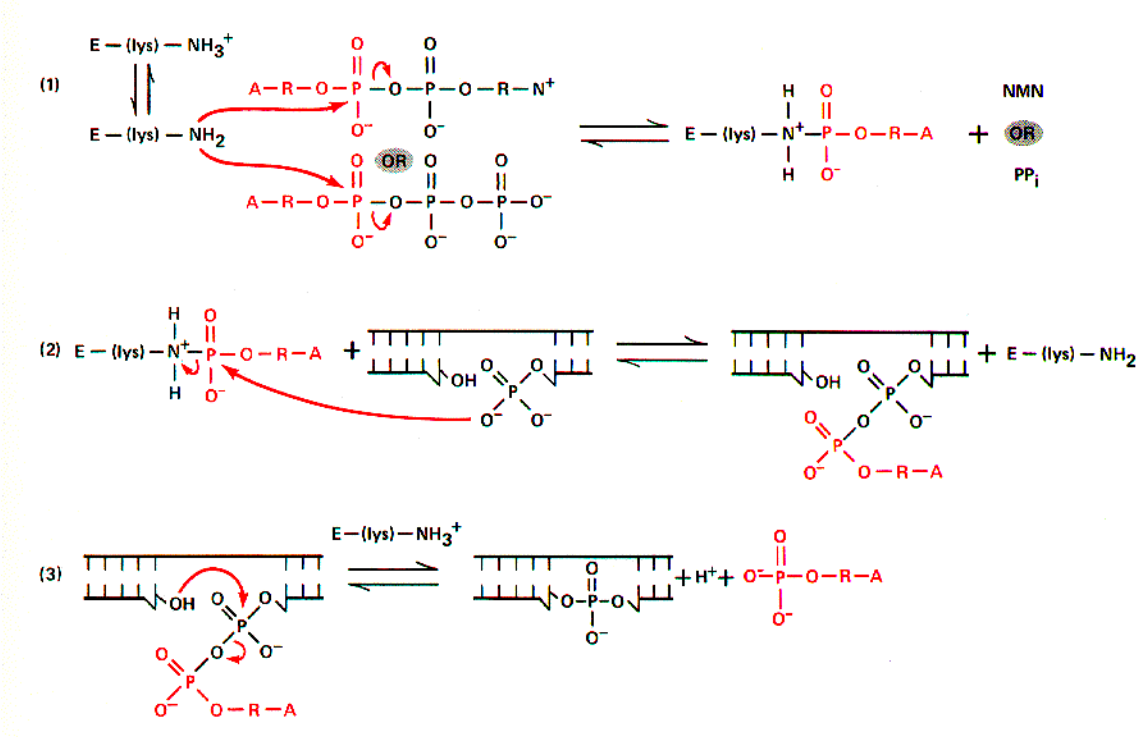
In the pathways to be discussed tomorrow, the recognition of the damaged base leads first to excision of the base, followed by recognition of the absence of a base by an enzyme called the apurinic endonuclease.

DNA Ligase Seals Nicks

Subsequent to the nick translation reaction, regardless of the mechanism of incision we are now left with a nick in the DNA. Nicks in DNA can be repaired by the enzyme DNA ligase.

DNA ligase from *E. coli* can repair the phosphodiester bond at a nick by using the energy in the pyrophosphate bond in NAD.

DNA ligase requires the presence of a 3'-hydroxyl group and a 5' phosphoryl group on the nick in order to seal the nick. DNA ligase catalyzes three reactions in order to seal a break as shown below.



The intracellular concentration of NAD and ligase are such that most of the free ligase will be in the charged form.

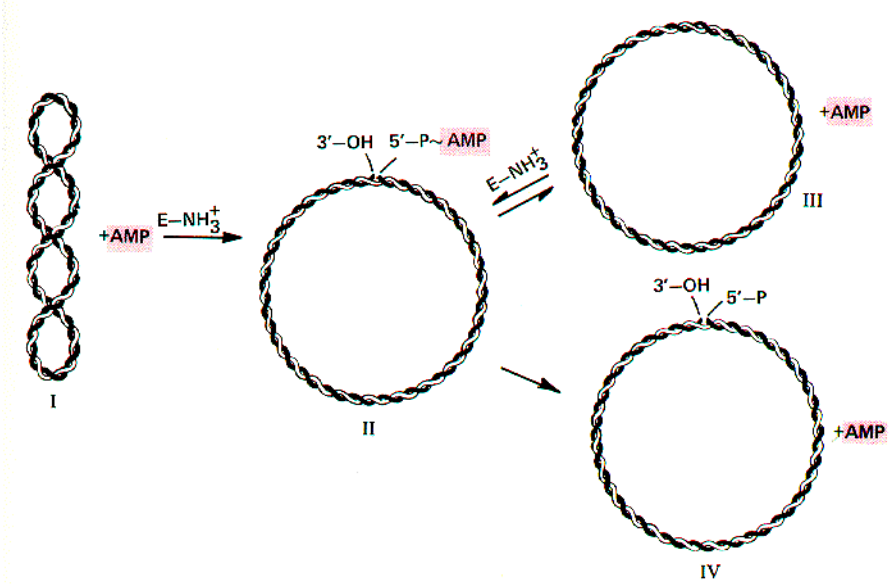
The ligase then transfers the adenyl group to the 5' phosphoryl group at the nick. This regenerates a pyrophosphate bond similar to that present in the NAD itself.

Then the ligase mediates the attack of the free 3' hydroxyl on this pyrophosphate bond that it just made, causing the release of AMP and leaving the phosphodiester bond closed.

Many other DNA ligases use a similar mechanism however, not all of them use NAD for the cofactor. Most eukaryotic ligases utilize ATP directly as the source of the adenyl group and they release pyrophosphate upon charging rather than NMN.

The DNA Ligase Reaction is Reversible.

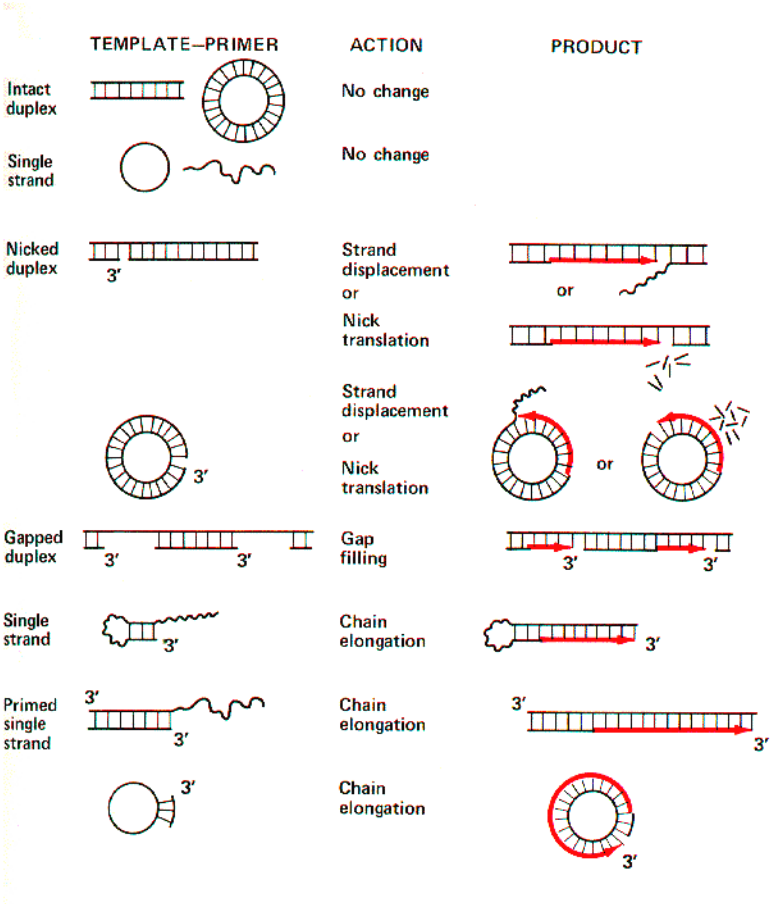
If a supercoiled DNA is incubated in the presence of uncharged DNA ligase and AMP, the DNA then becomes nicked, at a random site. The AMP is attached covalently to the 5' phosphoryl at the nick.



At this point the enzyme can continue the backward reaction and transfer the adenyl group to itself to become a charged AMP-ligase and leave the DNA in a nicked form, or it can now continue in the forward direction, resealing the DNA back to a covalently closed circular form.

The presence of the transient nick results in the relaxation of the supercoiled DNA. Therefore DNA ligase can act like an AMP dependent nicking-closing enzyme!

Summary of DNA Polymerase Action



Neither intact duplex circles nor intact duplex linear DNAs are template primers for DNA polymerase.

On the other hand a nicked circle or a nicked linear are good templates by virtue of the ability of DNA polymerase to mediate nick translation.

DNA polymerase can also extend a 3' primer terminus will displacing the 5' end ahead of the growing chain. This reaction is known as strand displacement.

In vitro nick translation is favored at low temperatures, temperatures that tend to stabilize the DNA helix from denaturation, while strand displacement is favored at 37°C.

Later, during a synthesis of this type, for reasons that are not completely understood, the enzyme can switch from copying the template strand to copying the DNA strand that it is displacing. This reaction is known as template switching.

DNA containing gaps is a good substrates with DNA polymerase first filling in the gaps, and then catalyzing nick-translation as above.

Even single-stranded DNA can serve as a substrate by virtue of very short transient base pairing within the DNA. As soon as a limited amount of pairing occurs and synthesis begins, the newly replicated DNA stabilizes the hairpin structure and the reaction can continue to the end of the template.

Discovery of Other DNA Polymerases

Because of the specificity of DNA polymerase (its inability to start DNA chains and its inability to extend 5' termini) several laboratories set out to look for other DNA synthetic enzymes. Between 1970 and 1972, two other enzymes were found in *E. coli* but they all catalyzed the same chemical reaction.

Table 5-1
Properties of polymerases I, II, and III of *E. coli*

	pol I[a]	pol II[a]	pol III[a] (core)
Functions			
Polymerization: 5′→3′	+	+	+
Exonuclease: 3′→5′	+	+	+
Exonuclease: 5′→3′	+	−	−
Pyrophosphorolysis and PP$_i$ exchange	+		+
Template primer			
Intact duplex	−	−	−
Primed single strands,[b]	+	−	−
stimulation by SSB	−	+	−
Nicked duplex (poly d(AT))	+	−	−
Duplex with gaps or protruding single-strand 5′ ends of: < 100 nucleotides	+	+	+
> 100 nucleotides	+	−	−
Polymer synthesis de novo	+	−	−

DNA polymerases II and III as they are called, both utilize 5' deoxynucleoside triphosphates and both of them extend only 3' primer termini.

Table 5-2
Major subunits and subassemblies of pol III holoenzyme

Subunit	Mass (kDa)	Gene	Subassembly			
α	130[a]	*dnaE*	pol III (core)	pol III′	pol III*	holoenzyme
ϵ	27.5[a]	*dnaQ* (*mutD*)				
θ	10					
τ	71[a]	*dnaX*				
γ	47.5[a]	*dnaX*	γ complex			
δ	35					
δ'	33					
χ	15					
ψ	12					
β	40.6[a]	*dnaN*				

[a] Based on DNA sequence; others are based on electrophoresis.

DNA polymerase II is very similar to DNA polymerase I except that it lacks a 5' exonuclease. It also appears to be unable to displace the 5' strand at a nick and therefore is completely inert on a nicked DNA template.

This enzyme works best on DNA that contains short single-stranded gaps.

DNA polymerase II does contain a 3' exonuclease which has exactly the same specificity for mismatched nucleotides as the 3' exonuclease of polymerase I.

DNA polymerase III also has a 3' exonuclease for proof reading and lacks a 5' exonuclease. It can synthesize DNA at a much greater rate than can either DNA polymerase I or II, rates approaching those of the replication fork (1-2000 nucleotides per second).

Mutants that are missing any one of these DNA polymerases have suggested that both DNA polymerase I and DNA polymerase III are essential for DNA replication. There are temperature sensitive lethal mutations in both enzymes.

There are other mutations in either DNA polymerase I or DNA polymerase II which are not lethal and which show an increased sensitivity to mutagenic agents and an inability to repair damage to DNA. The level of DNA polymerase II in the cell is also induced over 10 fold in response to damage in the DNA (part of the SOS response). Thus these enzymes may also play important and redundant roles in repair of damage to DNA.

Table 4-10
Viability of *polA* mutants with defects in 5′→3′ exonuclease

Mutant	Mutation	Defective in repair and joining of nascent fragments	Enzyme defect in vitro	Amino acid affected	Conditionally lethal
polA1	amber	yes	polymerization	trp342→am	no
polA12	ts[a]	yes	nick translation	unknown	no
polAex1	ts	yes	5′ → 3′ exo	gly184→asp	yes
BT4113	ts	yes	polymerization, 5′ → 3′ exo	gly103→glu	yes

[a] ts = temperature-sensitive.

One of the main problems with dissecting the functions of the enzymes of DNA metabolism by genetic studies has long been the redundant nature of all of the pathways for repair, replication, and recombination. When a cell has a multiplicity of choices open to it, eliminating one enzyme genetically often has little phenotypic effect.

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