

# Mechanistic Aspects of DNA Polymerases: *Escherichia coli* DNA Polymerase I (Klenow Fragment) as a Paradigm

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## I. Introduction

The DNA polymerases catalyze the accurate template-directed incorporation of the four deoxyribonucleoside 5'-monophosphate (dNMP) residues into a growing primer strand, in accordance with the Watson-Crick base-pairing rules. They fall broadly into two classes: those involved in bidirectional DNA replication at origins of replication in duplex chromosomes where they act as a member of a higher order protein assembly; and those that participate primarily without accessory proteins in DNA repair processes at gaps in the DNA. Despite the complexities of the initiation and replication events, the actual polymerization reaction features an enzyme able to catalyze template-directed polymerization in a 5' → 3' direction by nucleophilic displacement at the  $\alpha$ -phosphorus of a dNTP substrate by the 3'-hydroxyl of a complementary DNA or RNA

primer. The general features of the enzymology of prokaryotic and eukaryotic DNA replication have been summarized in extensive review articles or texts.<sup>1-4</sup>

For purposes of our review we will focus on what is known about the molecular structure and the dynamics of the polymerization reaction for these enzymes. At present, the material for this review then quickly reduces primarily to a wealth of studies on *Escherichia coli* DNA polymerase I discovered by Kornberg et al. some 35 years ago.<sup>5</sup> Limited proteolysis cleaves *E. coli* pol I into two fragments.<sup>6,7</sup> The large C-terminal fragment, termed the Klenow fragment, contains the unchanged polymerase and 3' → 5' exonuclease activities. The smaller N-terminal fragment retains the 5' → 3' exonuclease active site. The Klenow fragment of polymerase I is the only polymerase for which there is available a structure based on crystallographic data coupled to detailed kinetic analysis. Fortunately, in many but not all aspects the insight derived and characteristics inferred from investigations on this DNA repair enzyme appear in outline to encompass repair proteins from other species and to extend to replicative enzymes as well. Our emphasis will be on the molecular and dynamic features of the Klenow fragment, particularly those that contribute to the fidelity of the copy although appropriate comparisons will be introduced when the data warrant.

## II. Structure

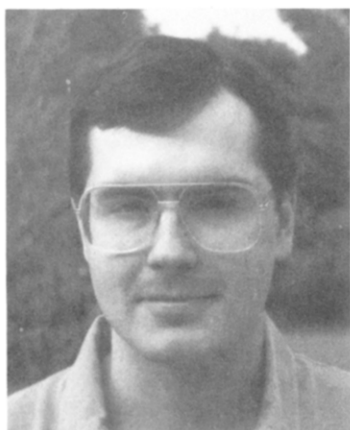
### A. X-ray Crystallography

The three-dimensional structure of Klenow fragment<sup>8</sup> reveals that the 605 amino acid polypeptide is folded into two distinct structural domains of approximately 200 and 400 amino acids (Figure 1).

The larger C-terminal domain contains a very deep cleft—20–24 Å wide and 25–35 Å deep—that binds duplex DNA and contains the active site for polymerization. Firm evidence for this contention is derived from several lines of evidence: measurements of fluorescence energy transfer, emission and anisotropy that are sensitive to the cavity's dimensions (see section II.C); electrostatic calculations describing a large positive attractive field within this cleft;<sup>9</sup> mutagenesis within the helix K that weakens the enzyme's interaction with DNA;<sup>10,11</sup> chemical modification of side chains within the cleft (section II.B) and directly from cloning and isolation of the C-terminal domain.<sup>12</sup> The isolated C-terminal fragment has a higher  $K_M$  for both duplex and dNTP substrates but a similar turnover number to the intact Klenow fragment. Although no crystal-



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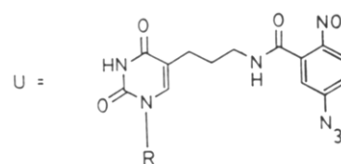


Steven S. Carroll received his B.S. in chemistry from the University of Virginia, where he worked in the laboratory of Raja Khalifah studying carbonic anhydrase. He carried out his graduate research at the California Institute of Technology in John Richards' laboratory, studying coenzyme binding to diol dehydratase and site-specific mutants of  $\beta$ -lactamase. His postdoctoral work was carried out in the laboratory of Stephen J. Benkovic studying the fidelity of DNA replication as catalyzed by mutants of polymerase I.

lographic structure is yet available for a polymerase-DNA complex, model building consistent with this evidence places double-stranded B DNA within the cleft and contacting the J and K  $\alpha$ -helices in a major groove.<sup>8</sup>

**CHART I. DNA Substrates Containing Base Modified with a Photoprobe**

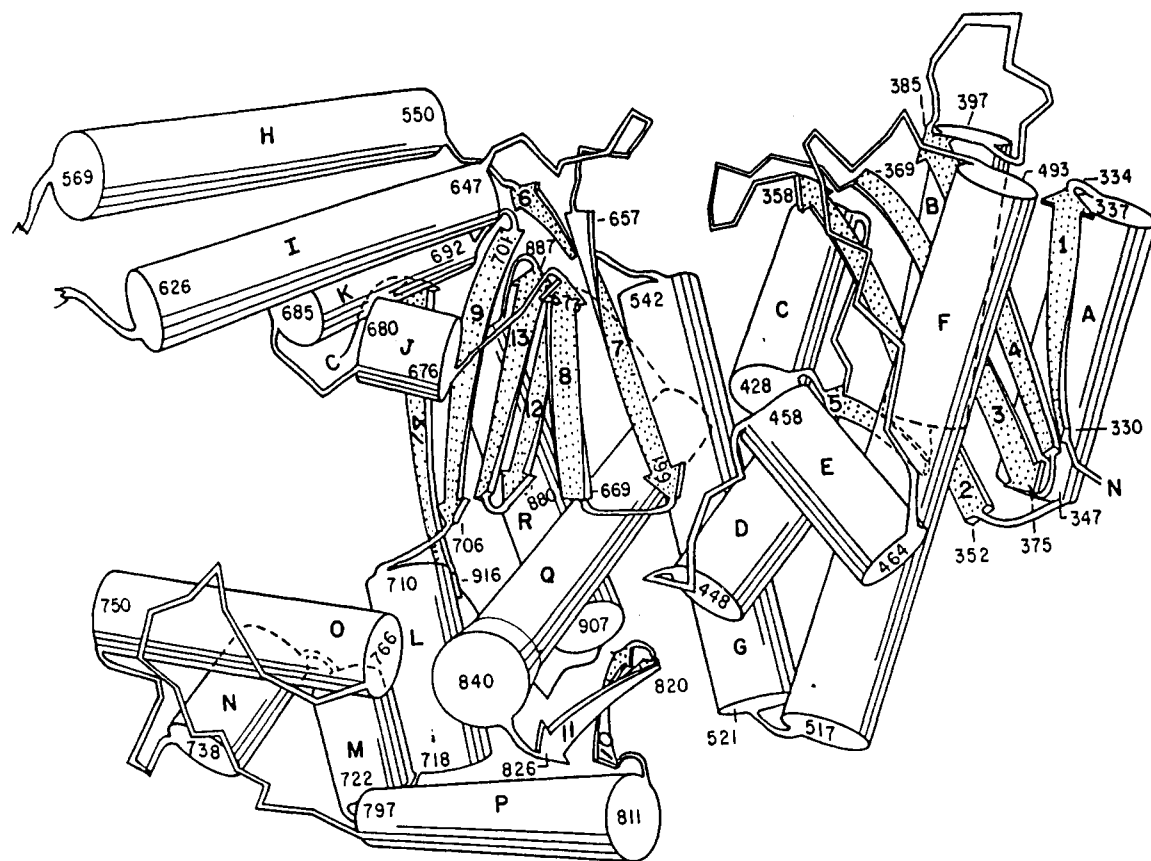
11az/20mer	3' AGCGTCGGCAGGT TCCCAAA 5'	5' TCGCAGCCGUC 3'
10az/20mer	5' TCGCAGCCGU 3'	
12az/20mer	5' TCGCAGCCGUCC 3'	
14az/20mer	5' TCGCAGCCGUCCAA 3'	
17az/20mer	5' TCGCAGCCGUCCAAGGG 3'	
20az/20mer	5' TCGCAGCCGUCCAAGGGTTT 3'	
13/20mer	3' AGCGTCGGCAGGT TCCCAAA 5'	5' TCGCAGCCGTCCA 3'



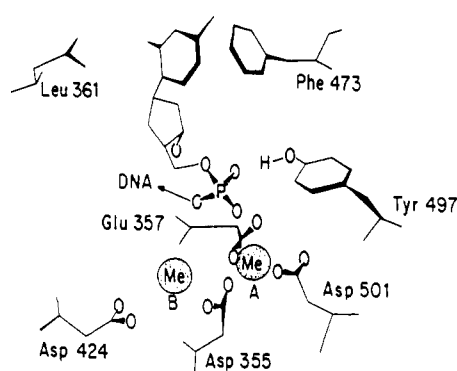
The flexible helices H and I can be imagined to fold across the binding cleft; data bearing on this conjecture also will be presented later in this section. Residues 570–625 of the polymerase domain connecting helices H and I are not sufficiently well resolved in the crystal to be defined in the structure. These residues also may act to close off the outside of the binding cleft when DNA is bound.

The smaller N-terminal domain has a central core of  $\beta$ -pleated sheet with  $\alpha$ -helices on both sides and a dNMP bound in the region of two divalent metal ions (Figure 2). One metal site is coordinated by the carboxylate groups of Asp-355, Glu-357, and Asp-501 together with the phosphate of dNMP. A second divalent metal ion is located between the dNMP phosphate and the carboxylate of Asp-424. Since deoxynucleoside monophosphates inhibit the 3'–5' exonuclease reaction,<sup>14</sup> the exonuclease activity has been ascribed to this region. Site-directed mutagenesis to yield two mutant proteins in which Asp-424  $\rightarrow$  Ala or Asp-355 and Glu-357  $\rightarrow$  Ala confirmed this locus since the activity of both was reduced by about 5 orders of magnitude.<sup>15</sup> Satisfyingly, difference electron density maps of the two mutant proteins showed no significant conformational alterations in either protein compared to the wild type. The fact that the double-mutant protein failed to bind either dNMP or metal ions whereas the single-mutant protein bound both dNMP and metal ion at site A (Figure 2) implicates site B in catalysis. Neither mutation had any measurable effect on the polymerase activity, indicating that the two sites must be separate to some degree.

Cocrystallization of the Klenow fragment with an 8-bp (bp = base pair) duplex gave rise to a difference electron density map showing three to four bases of ordered single-stranded DNA bound at the 3'  $\rightarrow$  5' exonuclease active site.<sup>16</sup> The rest of the DNA curiously was not visible in the electron density map, although numerous conformational changes were seen, particularly movement of the H and I helices that make up one side of the DNA binding cleft. The relationship, if any, between the observed changes in the protein structure



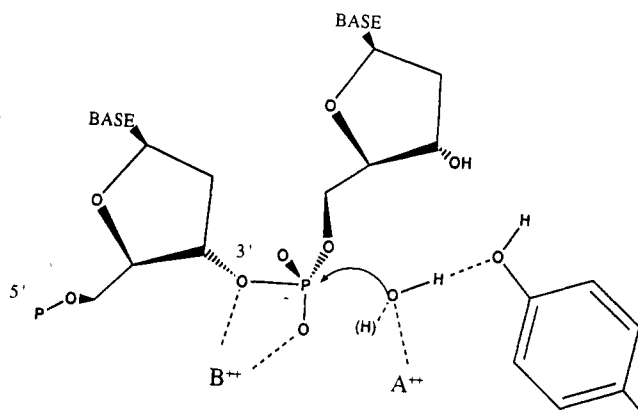
**Figure 1.** Structure of the Klenow fragment of *E. coli* DNA polymerase I (reprinted from ref 8; copyright 1985 Macmillan).



**Figure 2.** Structure of the 3'  $\rightarrow$  5' exonuclease active site of the Klenow fragment (reprinted from ref 13; copyright 1987 Elsevier).

and binding of single-strand DNA in the 3' exonuclease active site is not clear. Diffusion of p(dT)<sub>4</sub> into crystals of the two mutant forms of the protein described earlier produced complexes whose difference maps showed an extended conformation of the single strand in the exo site with the protein side chains contacting the 3'-terminal three bases and forming a series of apparent hydrogen bonds with the sugar-phosphate backbone.

From the structure of these DNA-Klenow fragment complexes, only the two divalent metal ions and the  $\gamma$ -OH of Tyr-497 are in close enough contact with the phosphodiester targeted for cleavage to be involved in catalysis. Since hydrolysis results in inversion of configuration at phosphorus,<sup>17</sup> the tyrosine cannot participate covalently in the cleavage process. The metal ions may act to stabilize a transient pentacoordinate species as well as facilitate the attack of water or a hydroxide molecule (Figure 3).

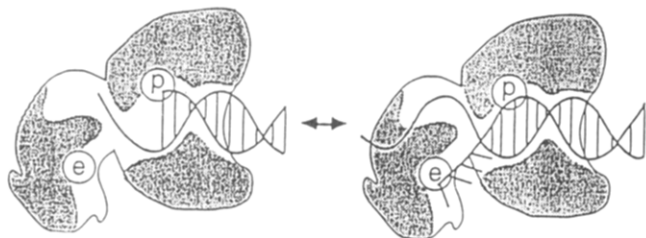


**Figure 3.** Proposed mechanism of the 3'  $\rightarrow$  5' exonuclease activity (reprinted from ref 28; copyright 1988 National Academy of Sciences).

## B. Chemical Modification

The results of classical chemical modification studies employing a variety of semispecific labeling reagents have independently corroborated the loci of the DNA and dNTP binding sites inferred from the X-ray structures.

Inactivation of the Klenow fragment with pyridoxal 5'-phosphate (PLP) treatment results in the labeling of four PLP-sensitive sites.<sup>18,19</sup> The presence of either deoxynucleoside triphosphate or template primer protects the enzyme against modification of one PLP-sensitive site, although the residue labeled differs in the two cases. Tryptic digestion of the labeled enzyme followed by resolution and sequencing of the peptide fragments led to the identification of Lys-635 (template



**Figure 4.** Proposed mechanism of the translocation of DNA from polymerase active site to exonuclease active site (reprinted from ref 28; copyright 1988 National Academy of Sciences).

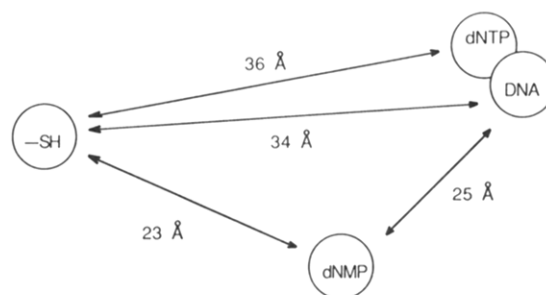
primer site) and Lys-758 (dNTP site). The former is located in the I helix and the latter is in the O helix, both within the duplex binding cleft. By similar protocols, Arg-841 in the Q helix was specifically trapped with phenylglyoxal<sup>20</sup> with the loss of polymerase activity. Histidine-881 in the  $\beta$ -sheet was cross-linked albeit in low yield by dTTP activated by ultraviolet light.<sup>21</sup>

The accuracy of modification experiments is generally much improved by using reagents that mimic the substrates. Affinity labeling with 5'-(fluorosulfonyl)-benzoyl-adenosine, an ATP analogue, led to its attachment to Arg-682 with loss of polymerase activity.<sup>22</sup> Both substrate and template primer protect this site, which is located between the J and K helices. Cross-linking with 8-azido-dATP labels Tyr-766<sup>23</sup> on the O helix on the opposite side of the cleft. This same tyrosine is also modified with a short DNA duplex containing the photoactivatable azido probe shown in Chart I.<sup>24</sup> Since the 11 az/20 mer is an efficient substrate for the polymerase activity of the Klenow fragment, it is very likely that Tyr-766 is proximal to the 3' primer terminus and necessarily adjacent to the dNTP binding site. A homologous tyrosine appears in the aligned primary sequences of other phage and bacterial polymerases.<sup>8,25-27</sup> In general, the results of these chemical modification experiments are in accord with the gross features of the DNA complex that has been model-built into the X-ray structure of the protein. However, by their very nature these reagents in most cases are imprecise tools so that the amino acids actually participating in the polymerization process may not be identified. As in the case of the 3'  $\rightarrow$  5' exonuclease site, genetic methods of mutagenesis will better serve in the identification.

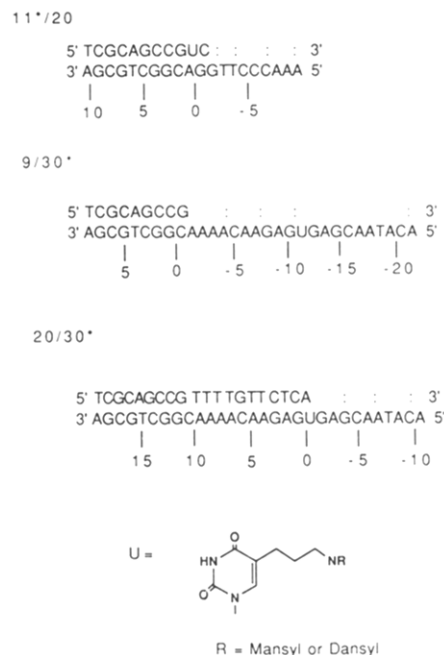
### C. Probes Based on DNA

The X-ray crystallographic data and domain experiments support a structure in which the polymerase and 3'  $\rightarrow$  5' exonuclease sites are 25–30 Å apart. An intramolecular "sliding" pathway involving four nucleotides of duplex DNA and four nucleotides of "melted" single-stranded DNA has been proposed to span between the locus<sup>28</sup> of the polymerase active site (p) and 3'  $\rightarrow$  5' exonuclease activity (e), as depicted in Figure 4.

Direct evidence that the solution structure of a ternary complex consisting of Klenow fragment, duplex DNA, and dNMP possessed intracomplex dimensions closely approximating those derived from the X-ray crystal structure was obtained by fluorescence energy-transfer experiments.<sup>29</sup> Fluorescent derivatives<sup>30</sup> of duplex DNA, dNTP, and dNMP modified with aminonaphthalenesulfonates served as energy-transfer do-

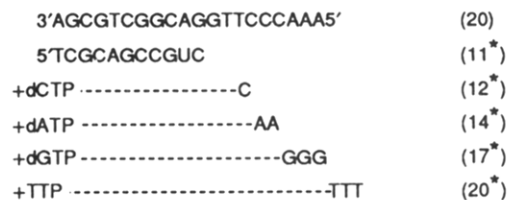


**Figure 5.** Distances between binding sites on the Klenow fragment.



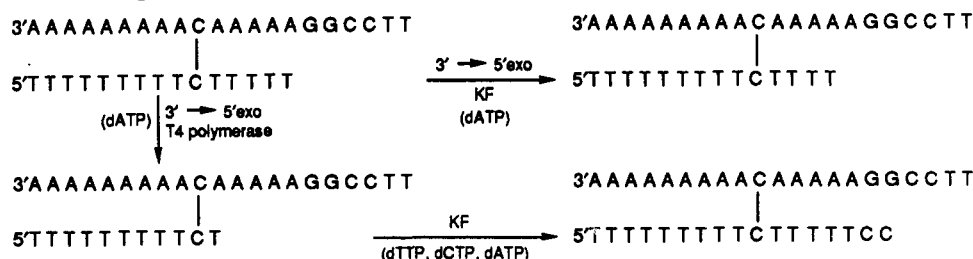
**Figure 6.** DNA substrates containing a base modified with a fluorescent probe.

nors to the fluorophore used to modify cysteine-907, 4-[N[(iodoacetoxy)ethyl]-N-methylamino]-7-nitro-benz-2-oxa-1,3-diazole. Pertinent structures are given below:



U is defined in Figure 6

The labeling of cysteine-907 caused no decrease in the enzyme's polymerase activity. The addition of dNTPs in the order indicated allowed extension of the primer terminus but did not produce significant changes in the measured sulfhydryl to DNA distance even though the fluorophore was moved a distance of almost one complete turn of DNA. After these measurements were compared with those from other donor-acceptor pair combinations, a distance separation map was constructed that is in satisfactory agreement with dimensions measured directly from the X-ray crystallographic structure. Thus, there is little conformational change that would act to bring the two domains together (Figure 5).

SCHEME I. Cross-Linked Duplex<sup>a</sup>

<sup>a</sup> dATP to prevent 3'  $\rightarrow$  5' degradation.

Fluorescent oligonucleotides in which the derivatizable base is placed at varying positions within the template or primer strand have been employed to map the Klenow fragment duplex contacts.<sup>31</sup> The duplex systems are listed in Figure 6 and designated 11\*/20, 9/30\*, and 20/30\* mer. The addition of Klenow fragment generally causes a blue shift and an increase in the fluorescence emission that is more pronounced when the fluorophore is within one base of the primer terminus.

Elongation of the 11\*/20 mer in discrete steps by addition of the appropriate dNTP led to a decrease in the fluorescence emission intensity as well as a red shift, indicating that the fluorophore is moving into a more hydrophilic environment. At position +7 the intensity or anisotropy of the emission spectra is similar to that seen with uncomplexed duplex in accord with the cleft region binding between five and eight base pairs and is equivalent to that obtained from DNA footprinting experiments.<sup>11</sup>

To observe protein-DNA interactions in the single-strand region of a primer template, the 9/30\* and 20/30\* systems were studied. The stronger contact points led to two maxima in the fluorescent emission spectra, one at position -11 and a second at position -4. An emission minimum occurred at position -7. Collectively, the emission data suggest a helical property of the single-stranded template when bound to the enzyme. Furthermore, at position +10 there was still enhanced emission (3-fold) from the fluorophore when in the template but not in primer strand. This difference has been attributed to movement of the H and I helices down across the occupied cleft.

Additional evidence that the polymerase and 3'  $\rightarrow$  5' exonuclease sites are separated was supplied by the behavior of the Klenow fragment toward a series of biotinylated DNA duplexes (Figure 7a) and adenosine 2',3'-epoxide triphosphate.<sup>32</sup>

The addition of avidin to biotinylated DNA provided duplexes having a very large group attached to a specific base.<sup>33</sup> This bulky group should be able to impede the activity of Klenow fragment by blocking its access to the primer terminus. It was found that the enzyme could extend duplexes where the primer terminus was six or more bases away from the base containing the biotin-avidin adduct, whereas the exonuclease site of the enzyme could only remove nucleotides from the primer terminus if the primer terminus was fifteen or more bases from the base with the biotin-avidin adduct. Thus, compared with the polymerase site, the exonuclease site requires nine more base pairs between the primer terminus and the base with the biotin-avidin complex.

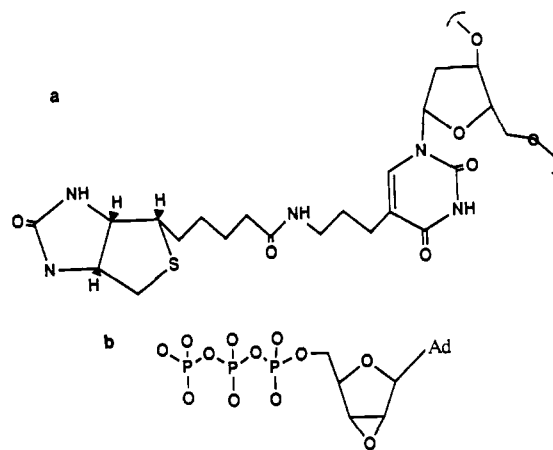


Figure 7. Structures of a biotinylated base (a) and epoxy-ATP (b).

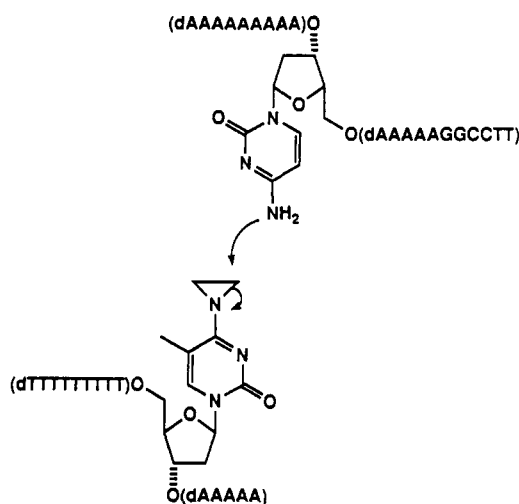
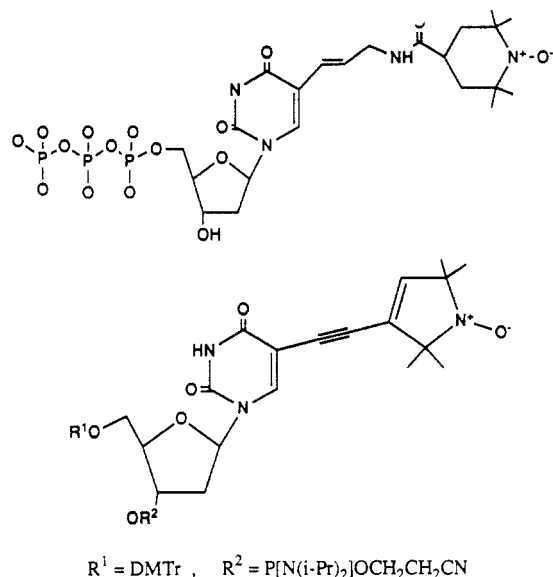


Figure 8. Proposed mechanism of cross-link formation.

Epoxy-ATP (Figure 7b) inactivates the Klenow fragment in a time- and concentration-dependent manner. Concomitant with inactivation is the incorporation of epoxy-AMP into the primer strand. Inactivation of the enzyme results from slow dissociation of the intact epoxy-terminated template primer from the enzyme. While the polymerase activity of the enzyme is suppressed by epoxy-ATP, the 3'  $\rightarrow$  5' exonuclease activity remains intact, strongly supporting the proposal that the two sites are spatially distinct.

A second type of chemically modified DNA duplex was created to test the hypothesis that strand separation was required to access the exonuclease site. A cross-linked duplex was synthesized by annealing primer and template strands, with the former bearing an aziridine at the 4-position of a cytosine base (Figure



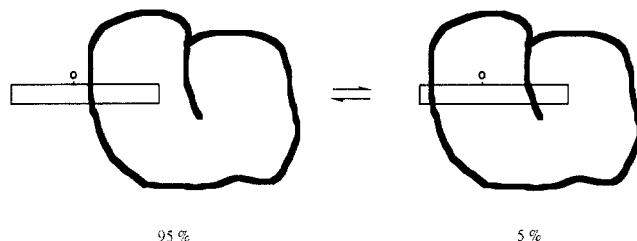
**Figure 9.** Structures of bases modified with nitroxide spin labels.

8) and the latter a reactive 4-amino group of the cytosine.

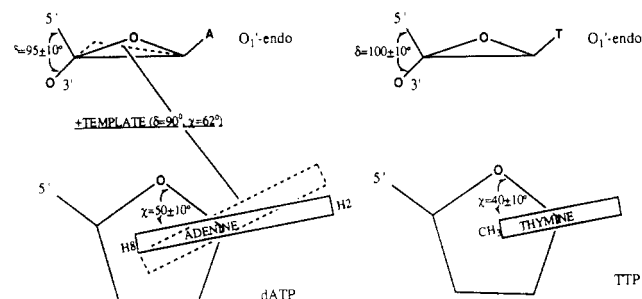
The action of Klenow fragment on the cross-linked duplex is summarized in Scheme I. These results demonstrate that the polymerase site of Klenow fragment does not require that the DNA strands be separated to act as a substrate since extension of 3'-terminus occurred from a nucleoside once removed from the interstrand crosslink. In contrast to the polymerase site, the exonuclease site requires that the primer terminus be at least four bases from the cross-linked base pair. On the other hand, the 3'  $\rightarrow$  5' exonuclease activity of the T4 polymerase requires a strand separation of two rather than the four or five bases required by the Klenow fragment.

The fluorescent probes as well as those recently reported that contain nitroxide spin labels tethered to the 5-position of the uracil ring (Figure 9)<sup>34-36</sup> offer the opportunity upon incorporation into DNA for monitoring the local dynamics of the duplex. When bound by Klenow fragment and examined by the use of time-resolved fluorescent spectroscopy, the decay behavior is a very sensitive probe of the local probe environment.<sup>37</sup> With the 11\*/20 mer described above, the decay of fluorescence anisotropy was accelerated from a half-life of approximately 80 ps when the modified base was outside of the KF binding cleft to <8 ps when the probe was between five and seven bases upstream from the primer 3'-terminus, presumably in contact with the enzyme. This cleft size is in accord with the steady-state fluorescent measurements. The decrease in the lifetime of the decay of fluorescence may be due to changes in the electrostatics of the two environments.<sup>37</sup> Moreover, the decay of anisotropy with the probe at positions +7 and +10 exhibited a nonmonotonic time dependence. This arises from two bound substrate configurations (Figure 10) distributed ca. 95% (out) and ca. 5% (in).

In one configuration, the DNA is bound with the primer 3'-terminus at the polymerase site, in which case substrate positions +7 and +10 are exposed to the solvent. In the other configuration, the DNA is bound such that positions +7 and +10 lie within the protein structure. The binding of the primer 3'-terminus at the



**Figure 10.** Position of the fluorescent probe when the primer terminus is bound at the polymerase active site (left) or at the 3' exonuclease active site (right).



**Figure 11.** Conformations of nucleotides when bound to the Klenow fragment in the presence (dotted lines) or absence (solid lines) of templates oligo(rU)<sub>54±11</sub> or oligo(rA)<sub>50±13</sub>. Adapted from ref 38.

3'  $\rightarrow$  5' exonuclease site would generate such a configuration according to the "slide and melt" model for substrate translocation under discussion since an eight-base-pairs movement is required for relocating the primer 3'-terminus from the polymerase to the 3'  $\rightarrow$  5' exonuclease site. Direct support for this proposal was obtained from duplex complexes with the *exo*<sup>-</sup> double mutant, which does not bind DNA substrate at the 3'-5' exonuclease active site. This protein did not exhibit the nonmonotonic anisotropy decay with bound and elongated 11\*/20 mer. Thus, this sensitive technique provides a unique picture of the substrate translocation event within the Klenow fragment.

## D. Probes Based on dNTP

Nuclear Overhauser effects (NOE's) have been used to determine interproton distances and conformations of substrates bound to the Klenow fragment, in the absence and presence of poly(rA) and poly(rU) templates.<sup>38,39</sup> Oligoribonucleotides were chosen as templates over blocked oligodeoxyribonucleotides because of their enhanced stability. Moreover, they function as substrates. From the time dependence of the NOE's between H's on the deoxyribose and the heterocyclic base, models were constructed for the conformations of the bound nucleotides.

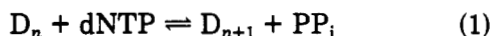
The conformation of dATP in the presence and absence of template (poly(rU)) is depicted in Figure 11. In both the presence and absence of template the dATP adopts a conformation similar to that found for nucleotides in B DNA.<sup>40</sup> A similar conformation was deduced for TTP in the presence of poly(rA). For dGTP bound in the presence or absence of oligo(rU) and therefore mispaired, a 60:40 ratio of anti to syn conformations was observed. A mock quarternary complex, assembled from adenosine 5'-( $\alpha,\beta$ -methylene triphosphate), poly(rU)-(Ap)<sub>6</sub>A, and enzyme, showed that the interprotonic distances in the nucleotide analogue



were similar to those of dATP. In accord with the results presented in section III, the binding affinities of all these nucleotides for the Klenow fragment were within a 5-fold factor, suggesting that this nucleotide conformation is prior to one in the chemically active complex and may be representative of the nucleotide in the initial E-DNA-dNTP complex.

### III. Kinetic Mechanism of Polymerases

The overall reaction catalyzed by all polymerases, as described in eq 1, represents a summary of many discrete processes. The polymerase must bind at or near the 3'-hydroxyl of the substrate DNA in the presence of an enormous excess of duplex DNA and also bind the one correct dNTP in the presence of the other three incorrect dNTP's. Formation of the chemical bond



then occurs by nucleophilic attack of the 3'-hydroxyl of the growing primer strand on the  $\alpha$ -phosphorus of the dNTP, complexed with a divalent metal ion, generally  $Mg^{2+}$ . The polymerase then releases pyrophosphate and either translocates to the next available template position or dissociates from the substrate DNA. The ordering of  $PP_i$  release and translocation/dissociation may be simultaneous, random, or strictly sequential. Those polymerases possessing 3'  $\rightarrow$  5' exonuclease activity have the additional complication of the possibility of hydrolysis of the 3'-terminal base at one or more places in the reaction mechanism. Additionally, relatively modest levels of  $PP_i$  ( $\sim 50 \mu M$ ) have been shown to be capable of reversing the overall reaction, as catalyzed by KF, to regenerate dNTP and  $D_n$ .<sup>45</sup> (An alternative mode of chemical bond formation has been postulated to involve the formation of dNMP and  $PP_i$  prior to the incorporation into the primer strand, based on the decreased fidelity observed on addition of  $PP_i$  and on  $PP_i$  exchange with an incorrect base.<sup>41-43</sup> However, prior hydrolysis would result in the retention of configuration at the  $\alpha$ -phosphorus, but inversion of configuration has been observed experimentally.<sup>44</sup> Thus, the simplest explanation of observed results is a single nucleophilic attack at the  $\alpha$ -phosphorus.)

The complex interplay of the various processes required for synthesis of DNA and the extremely high accuracy demanded by organisms in copying their genetic material are in constant conflict with the rapidity demanded for synthesis. For example, phage T4, on infection of a host cell, makes 200 copies of its  $10^5$ -bp genome in about 20 min.<sup>46</sup> During their 4-min-long S phase, embryonic *Drosophila melanogaster* cells copy  $10^8$  bases.<sup>47</sup> To define completely the kinetic mechanism for correct incorporation by any polymerase, it is necessary to understand the rate of all separate reactions involved in the process of DNA synthesis. This discussion of kinetic experiments is divided into two main sections: those experiments involving steady-state methods with substrate DNA in excess of enzyme and those utilizing pre-steady-state methods to examine bursts of reaction on mixing enzyme and substrate. As with the structural studies, the most complete information on the kinetics of DNA synthesis is available for KF and the discussion will center on that polymerase.

### A. Steady-State Experiments

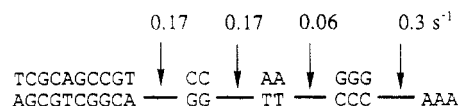
The great wealth of data on the kinetics of DNA synthesis for most polymerases has been derived by steady-state methods. These techniques have the important advantages of requiring relatively little enzyme, which is fortunate considering the difficulties involved in obtaining large amounts of some polymerases, particularly the protease-sensitive eukaryotic replicative polymerases,<sup>48</sup> and also requiring only standard laboratory equipment. However, as described below, steady-state methods have the distinct disadvantage of generally observing more than one of the separate kinetic processes involved in DNA synthesis, which necessarily complicates the interpretation of observations. For example, all steady-state distributive syntheses must involve incorporation of dNMP and dissociation of the enzyme from product DNA if more than one turnover is being observed. Therefore, the observed rate may be due to some rate involved in incorporation of dNMP such as chemical bond formation, the dissociation rate of the elongated product, or some combination of both rates. Indications of which process is rate-limiting can come from processivity measurements, but absolute proof requires independent measurement of both rates. Steady-state methods have proven useful in determining the kinetic ordering of substrate binding, the dependence of observed rates on DNA sequence, and processivity of DNA synthesis and can give some indication of the rate-limiting step(s) of synthesis.

#### 1. Kinetic Order of Binding

The binding of substrate DNA and dNTP can be either random, sequential, or strictly ordered. The order in which polymerase binds the substrate DNA and dNTP has been determined by three general methods.

The first method involves inhibition of polymerization by  $PP_i$  and indicates an ordered sequential binding scheme with mouse pol  $\beta$ <sup>49</sup> and pol I from *E. coli*<sup>50</sup> in which polymerase binds first to DNA and only afterward to dNTP, in a kinetically competent manner. Inhibition by  $PP_i$  with varying dNTP gave parallel lines on a double-reciprocal plot while inhibition by  $PP_i$  with varying DNA gave intersecting lines on the same plot. The mixed inhibition patterns observed on secondary plots indicated a mixed mode of inhibition where  $PP_i$  could form a dead-end complex with the E- $D_n$  species or could noncompetitively inhibit the E- $D_n$ -dNTP complex.<sup>51</sup> The overall conclusion from the  $PP_i$  inhibition patterns was a strictly ordered sequential binding first of DNA to pol  $\beta$ , followed by binding of dNTP.

A second method of determining the order of binding, isotope trapping,<sup>52</sup> has been used with pol I. The polymerase is incubated with one labeled substrate. A mixture of the second substrate and a large excess of unlabeled first substrate is then added to initiate the reaction. Observable reaction can only occur if the enzyme-first substrate complex is kinetically competent to react; otherwise, the bound labeled substrate is diluted by dissociation into the unlabeled pool. Isotope trapping experiments have shown that polymerase, though it can bind dNTP in the absence of DNA,<sup>53</sup> cannot do so in a kinetically competent manner. However, bound labeled DNA could be converted directly to product.



**Figure 12.** Product release rates at different positions of a duplex DNA substrate.

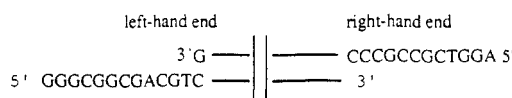
The third method of establishing the order of substrate binding has employed dideoxy-terminated primers<sup>54</sup> and KB cell DNA polymerase  $\alpha$ . Addition of the next correct dNTP to a complex of pol  $\alpha$  and a ddNMP-terminated primer template system led to the competitive inhibition of polymerization when the system was challenged by unblocked primer template, by formation of a dead-end complex: polymerase-ddNMP-terminated primer template-dNTP. Further, in separate experiments, pol  $\alpha$  was shown in a similar manner to associate first with the template prior to binding primer. Thus, its order of binding is first to template, then to primer, and then to dNTP. The authors argued that a preference for single-stranded template would ease the location of the primer terminus in the presence of an excess of nonreplicative duplex DNA.

All methods for examining the order of substrate binding to polymerases from a wide variety of sources have indicated a strictly ordered sequential binding mode of DNA before dNTP. Combined with the probable existence of only a single binding site for all four nucleotides,<sup>4</sup> these results indicate that strictly ordered sequential binding may be a general feature of polymerase action. Intuitively, efficient correct synthesis would require the initial binding of primer template since initial binding of dNTP could only be correct one out of four times.

## 2. Sequence Dependences for Observed Rates of Synthesis

Direct comparison of rates of synthesis by KF with poly(dA)-oligo(dT)<sup>52</sup> or cohesive ends of  $\lambda$  DNA<sup>55</sup> or short duplexes made from synthetic oligonucleotides<sup>45</sup> has revealed significant differences. Moreover, rates of synthesis under necessarily distributive conditions at different positions of one synthetic duplex are substantially different and are illustrated in Figure 12. Quantitative measurement of the binding constant for poly(dA)-oligo(dT) (200 nM<sup>52</sup>) and for "synthetic" 13/20 mer primer template (5 nM<sup>45</sup>) reveals a difference that may be due to the unusual bifurcated hydrogen-bonding network observed with runs of A-T base pairs,<sup>56</sup> and so it may not realistically represent the majority of natural DNA.

The DNA sequence can dramatically alter polymerase rates by altering the template structure. Pause sites can be identified as sites opposite which preferential accumulation of DNA chains of certain length occurs. Many pause sites can be correlated to positions of possible template secondary structure such as hairpin loops.<sup>57</sup> Stretches of both oligopurine, such as in poly(dA)-oligo(dT), and oligopyrimidine have been shown to be sources of pause sites<sup>58</sup> and may be caused by the same kinetically anomalous behavior as observed with runs A-T base pairs.<sup>52</sup> Pause sites from an M13 template system revealed similar pausing patterns for KF and pol  $\alpha$ .<sup>58</sup> The necessity of accessory proteins such as T4 gene 32 protein for kinetically competent synthesis may



**Figure 13.** DNA sequence at the ends of  $\lambda$  DNA.

be because of its ability to reduce deleterious template secondary structure.<sup>59</sup> Since KF covers up to 12 base pairs of duplex DNA<sup>23</sup> and an unknown amount of template sequence, structural changes anywhere along this stretch of DNA could potentially contribute to sequence-dependent differences in rate of incorporation, binding of DNA, or dissociation.

## 3. Processivity

The processivity of a polymerase is defined as the number of nucleotides incorporated on average during a single binding event to the primer template. Processivity may then be considered to be a competition between incorporation-translocation and dissociation of the primer template.<sup>60</sup> Values of processivity can range from 1, completely distributive, to hundreds or thousands of bases added. Four general methods have been developed for determining processivity: template challenge, nucleotide incorporation ratios, polymerization in the absence of one of the four dNTP's, and product separation and direct analysis.

Template challenge experiments employ two different primer templates. Generally, an excess of poly(dC)-oligo(dG) and dGTP are added to a reaction in which enzyme is actively replicating poly(dA)-oligo(dT). The length of time for cessation of incorporation of TTP is a measure of processivity. At 37 °C, this technique revealed for pol I an immediate decrease in incorporation of TTP.<sup>61</sup> However, during the mixing time of the experiment, the polymerase could have incorporated up to 100 nucleotides. At 4 °C, to decrease the rate of polymerization and with calf thymus DNA as challenge DNA, pol I was found to be distributive.<sup>50</sup> However, the anomalous behavior of pol I toward polynucleotide templates mentioned in section III.A.2 makes generalization of these results difficult.

The technique of determining nucleotide incorporation ratios takes advantage of the available template sequence at the cohesive ends of  $\lambda$  DNA as shown in Figure 13. If dGTP and dCTP are added to the reaction, the enzyme may extend the right-hand end of  $\lambda$  DNA by eight bases. If the polymerase operates processively, the ratio of incorporated dG to dC will always be 6:2 (3:1). If the polymerase acts distributively, then early during the reaction the ratio of dG incorporated to dC will be much greater than 3. At 6 °C, pol I was found to be completely processive for the template length of the right-hand end of  $\lambda$  DNA.<sup>62</sup> The obvious limitation of this technique is that processivities greater than 12 cannot be distinguished.

Comparison of the polymerization rates in the presence of all four dNTP's with the rate in the absence of one or more dNTP(s) can be used to determine processivity. This method requires the measurement of the ratio of incorporated label into DNA with and without the full complement of dNTPs and determination of the cycling time,  $T_x$ , the time it takes the polymerase to bind a primer terminus, extend it, dissociate from it, and diffuse to another primer terminus.<sup>63</sup> These experimentally determined parameters may then be

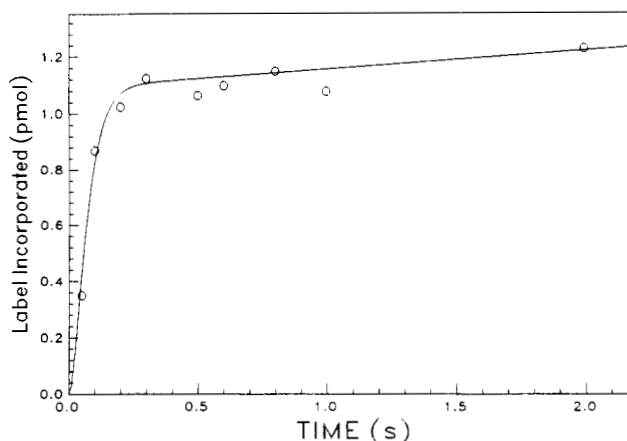


used to determine processivity from theoretical curves. This method has been useful in demonstrating the variations in processivity for a given polymerase under different reaction conditions. For pol I, processivity was as high as 188. Higher ionic strength and lower temperatures decreased processivity, such that at 37 °C and ionic strength 0.85 M, processivity was 40–50 with a gapped DNA substrate. With poly(dA) as template, processivity could vary from 3.3 to 188 bases depending on reaction conditions. Use of this method with KB cell pol  $\beta$  revealed it to be completely distributive under all conditions examined. An important caveat with this method is to show that the average available template length exceeds the measured processivity.

Another method of measuring processivity involves use of poly(dA)-oligo(dT) as primer template and adding [ $^3\text{H}$ ]TTP to extend the primers.<sup>64</sup> The extended primers are then isolated from template by chromatography on oligo(dT)-cellulose columns, digested with micrococcal nuclease and spleen phosphodiesterase. After separation by TLC and an ethanol wash, the ratio of [ $3'\text{-}^3\text{H}$ ]dTMP + [ $^3\text{H}$ ]dThd to [ $^3\text{H}$ ]Thd is calculated and equal to the processivity ([ $^3\text{H}$ ]dThd results from digestion of the last added base to a primer). By this method, T5 DNA polymerase was shown to be much more processive than pol I, T4 polymerase, calf thymus pol  $\alpha$ , or pol  $\beta$ . However, this method requires that each primer be extended only once or not at all during the reaction. This method revealed pol I to be 2–2.5 times as processive on “nicked-like” poly(dA) primer templates than on “gapped-DNA-like” poly(dA) primer templates, in contrast to the results of experiments comparing polymerization rates with and without all four bases.<sup>63</sup> The differences may be due to use of poly(dA) vs calf thymus DNA as substrates. A variation of the direct analysis method involves the use of a trap (such as calf thymus DNA) for free enzyme. The initiator,  $\text{Mg}^{2+}$ , and trap are added simultaneously, and then product DNA is analyzed either by filter binding if label is present as dNTP or by gel electrophoresis if substrate DNA is radiolabeled. (An important control for the experiment using radiolabeled dNTP is to monitor and subtract radiolabel incorporated into the trap.) This method revealed a 2-fold reduction in the processivity of Klenow fragment relative to intact pol I on poly(dA)-oligo(dT).<sup>52,68</sup> Overall, therefore, it is important when processivities are compared to bear in mind potential problems that might arise due to differences in reaction conditions such as temperature, ionic strength, or choice of substrate.

#### 4. Rate-Determining Steps from Steady-State Analyses

Indications of the complexity of kinetics of incorporation of an individual nucleotide have been noted from reactions employing steady-state conditions. Several investigators have noted that upon extrapolation of a steady-state reaction progress curve back to zero time an intercept on the product axis is obtained for pol I,<sup>50</sup> pol  $\beta$ ,<sup>65</sup> and T5 polymerase.<sup>66</sup> Biphasic kinetics are indicative of the participation of more than one kinetic event in the observed rate. Bursts proportional to the quantity of enzyme added as noted for pol I and T5 polymerase indicate a rapid initial incorporation followed by a slower rate that is rate-limiting in the steady



**Figure 14.** Biphasic reaction profile of incorporation of a single nucleotide into duplex DNA.

state. Quantitation of the burst rate requires pre-steady-state methods as discussed in the next section. Assignment of which step or steps in the mechanism correspond to which of the two experimentally observed rates requires additional experiments.

That the steady-state rate does not correspond to two of the many possible rates involved in incorporation of a nucleotide can be determined from the following experiments. Substitution of an  $\alpha$ -thio-dNTP for dNTP will result in a decrease of 30–100 if the actual chemical step is rate-limiting, based on comparison with model displacement reactions at phosphorus.<sup>67</sup> The observed decrease of only 2–3 in the steady-state rate indicates that the chemical step is not contributing to the observed rate. Another possibility may be ruled out on the basis of positional isotope exchange experiments using [ $\alpha\text{-}^{18}\text{O}$ ]dATP, labeled in both bridging and non-bridging positions. The lack of any observable exchange of  $\alpha$ - $\beta$  bridging  $^{18}\text{O}$  to  $\beta$  nonbridging  $^{18}\text{O}$  even in the presence of added  $\text{PP}_i$  suggests the release of  $\text{PP}_i$  is rapid and not likely to be involved in limiting the steady-state rate.<sup>68</sup>

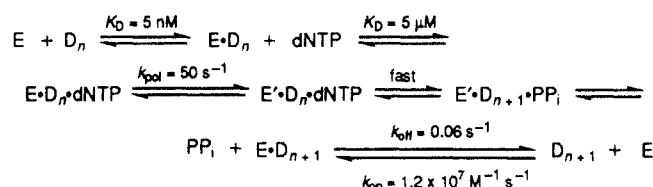
## B. Rapid Kinetic Methods

A complete understanding of the kinetic mechanism of KF requires the determination of the rates and equilibrium constants of all of the steps involved. Steady-state methods have helped to elucidate several important relationships between enzyme and reactants during the reaction. However, the isolation of individual rates and their quantitation requires the use of rapid kinetic methods. The advent of solid-support oligonucleotide synthesis, the development of an efficient overproduction system for KF,<sup>69</sup> and the design and construction of relatively simple instrumentation for rapid quench reactions<sup>70</sup> have allowed the application of rapid kinetic methods to the problem of the kinetic mechanism of KF.

### 1. Steps in the Forward Reaction

The use of synthetic DNA duplexes has provided a means of following the incorporation of a single nucleotide in a DNA substrate of KF, a process that is otherwise obscured by the presence of the other dNTPs. A typical reaction progress curve, shown in Figure 14, demonstrates the biphasic curvature of the incorporation reaction carried out with DNA in excess of KF.<sup>46</sup>

## SCHEME II. Kinetic Mechanism of Incorporation



The amplitude of the fast phase is stoichiometric with the amount of KF present, and the rate of the slow phase is the same as the rate of steady-state incorporation. Decreasing the concentration of dNTP decreased the rate of the fast phase but did not change the rate of the slow phase. Therefore, the biphasic reaction is consistent with a rapid step preceding a slow first-order process. These observations were interpreted to indicate a rapid incorporation of one dNTP per enzyme-bound DNA molecule followed by a slow dissociation of product DNA. The dissociation of product DNA is rate-limiting in the steady state and was found to vary from 0.06 to 0.6 s<sup>-1</sup> depending on the substrate.<sup>45</sup>

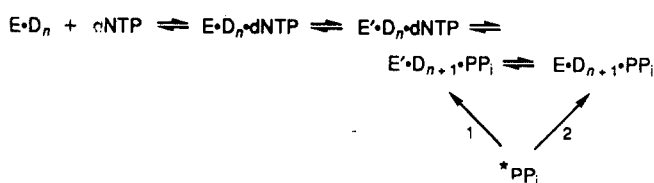
Substitution of  $\alpha$ -thio nucleotide created only a slight decrease in the rate of the fast phase, indicating formation of the chemical bond does not limit the rate of incorporation. In the absence of evidence to the contrary, the rate of the fast phase of incorporation was attributed to a rate-limiting conformational change of the E·D<sub>n</sub>·dNTP species. One possible physical explanation for this conformational change is a change in the coordination of Mg<sup>2+</sup>.<sup>39</sup>

Quantitative determination of the rates and thermodynamic binding constants associated with the mechanism as outlined in Scheme II has been carried out. The burst of incorporation was equivalent to that of enzyme-bound DNA. The steady-state turnover rates at various concentrations of DNA were measured, and the linear reactions were extrapolated back to time zero to determine the burst amplitude, which is equivalent to the amount of KF·DNA complex present prior to initiation of the reaction. Scatchard analysis of the bound vs free substrate gave a  $K_D = 5$  nM for DNA. The association rate of substrate DNA was calculated from the relationship  $K_D = k_{\text{off}}/k_{\text{on}}$  to be  $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . The  $K_D$  for dNTP and the rate of polymerization,  $k_{\text{pol}}$ , were determined by measuring the rate of the burst phase for varying concentrations of dNTP. Lineweaver–Burke replots of the data gave a  $K_M = 5 \text{ } \mu\text{M}$  (equivalent to  $K_D$  under these pre-steady-state conditions) and  $k_{\text{pol}} = 50 \text{ s}^{-1}$ .<sup>45</sup>

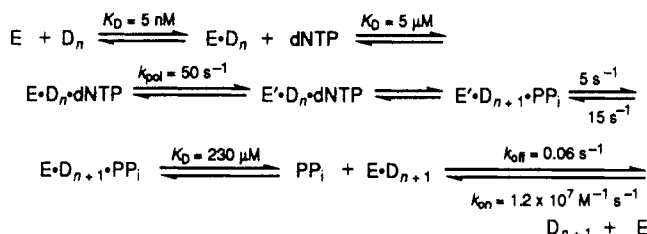
## 2. Work in Progress

An investigation of pyrophosphorolysis, the microscopic reverse of the incorporation reaction, has revealed more of the detailed energetics of the steps subsequent to formation of the E'·D<sub>n+1</sub>·PP<sub>i</sub> species.<sup>71</sup> The existence of a slow step following formation of E'·D<sub>n+1</sub>·PP<sub>i</sub> is inferred from the lack of any reproducible burst during pyrophosphorolysis. If there were no slow step following chemistry, which forms E'·D<sub>n+1</sub>·PP<sub>i</sub>, the back-reaction would be expected to show a burst because the rapid reverse of the chemical step would be followed by the slower reverse of the conformational change. The kinetic parameters of pyrophosphorolysis were determined by varying the concentration of PP<sub>i</sub> and measuring the rate of appearance of dNTP, giving

## SCHEME III. Pyrophosphate Exchange



## SCHEME IV. Expanded Kinetic Mechanism



a rate of 0.3 s<sup>-1</sup> and a  $K_D$  for PP<sub>i</sub> of 230  $\mu\text{M}$ .

Decay of the E'·D<sub>n+1</sub>·PP<sub>i</sub> species to released products may occur either through release of PP<sub>i</sub> followed by the conformational change that relaxes the resulting E'·D<sub>n+1</sub> species to E·D<sub>n+1</sub> or by prior relaxation to a E·D<sub>n+1</sub>·PP<sub>i</sub> species, followed by release of PP<sub>i</sub>. Experiments to determine which case obtains involve comparisons of the rate of pyrophosphorolysis with the rate of pyrophosphate exchange. Pyrophosphate exchange is measured as the rate of appearance of radiolabel into a pool of initially cold dNTP by addition of radiolabeled [<sup>32</sup>P]PP<sub>i</sub>. The pool of dNTP becomes radioactive because of an initial formation of extended product, D<sub>n+1</sub>, and then reversal of the incorporation by pyrophosphorolysis, as described in Scheme III.

If case 1 holds, PP<sub>i</sub> can exchange with the E'·D<sub>n+1</sub> species, avoiding the slow reverse of the conformational relaxation step from E·D<sub>n+1</sub>·PP<sub>i</sub> to E'·D<sub>n+1</sub>·PP<sub>i</sub>, and one would expect pyrophosphate exchange to occur at a faster rate than pyrophosphorolysis, which must traverse the conformational change. Since the measured rates of pyrophosphorolysis and pyrophosphate exchange are the same, case 2 must hold and the second conformational change from the E'·D<sub>n+1</sub>·PP<sub>i</sub> species to E·D<sub>n+1</sub>·PP<sub>i</sub> takes place before PP<sub>i</sub> can be released.

The expanded version of the kinetic mechanism is given in Scheme IV. The rate of the forward direction of the second conformational change converting the E'·D<sub>n+1</sub>·PP<sub>i</sub> species to E·D<sub>n+1</sub>·PP<sub>i</sub> was determined by measuring the rate of addition of the second correct dNTP to the duplex since the complex must go through that step prior to the second addition and was found to be 15 s<sup>-1</sup>. To maintain the observed net rate of pyrophosphorolysis, the reverse rate of the second conformational change must be set at 5.4 s<sup>-1</sup>. The rates of the chemical steps interconverting the E'·D<sub>n</sub>·dNTP and E'·D<sub>n+1</sub>·PP<sub>i</sub> species are assumed to be very fast to account for the lack of an  $\alpha$ -thio nucleotide effect on the burst rate. The equilibrium between these two species will be estimated by pulse-chase experiments. The contribution of the conformational changes before and after the chemical step to fidelity will be discussed in section IV.C.

The kinetic mechanisms of polymerases from phages T4 and T7 are currently under investigation by rapid kinetic methods.<sup>72,73</sup> Both phage polymerases show bursts of incorporation of dNTP into synthetic du-

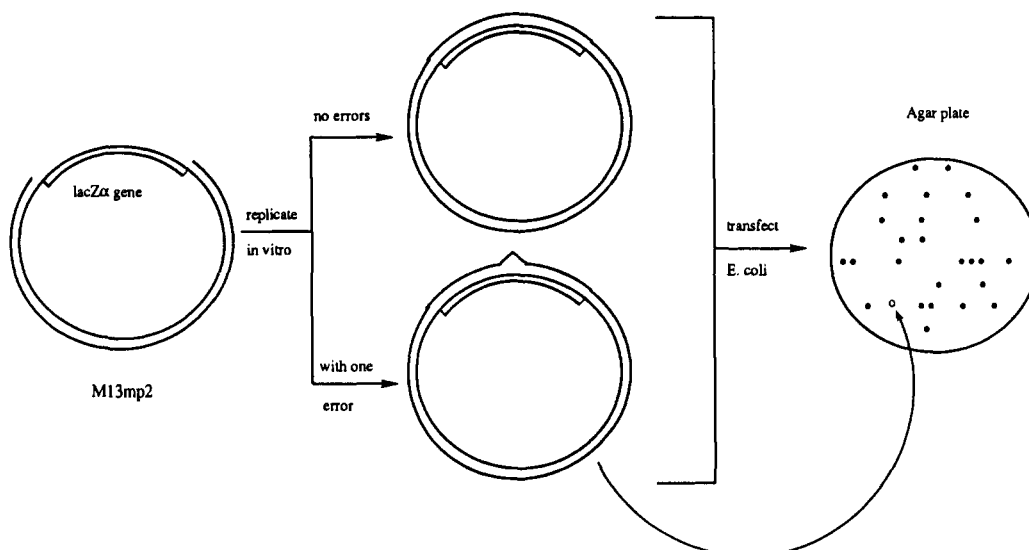


Figure 15. In vitro reversion and forward mutation assays.

plexes, arguing for some degree of similarity in their kinetic mechanisms compared to that of KF. Quantitatively, however, the rates of incorporation by T4 and T7 polymerases are much faster than that of KF, as are the rates of exonuclease activity of T4 and T7 polymerases. The diversity of the sources of these three polymerases argues that some features of polymerase activity may be general, including the rapid incorporation of dNMP and slow release of product DNA. The ratio of these rates may largely define the processivity of a given polymerase.

#### IV. Fidelity

The remarkably low rates of mutation observed in many organisms are the result of several processes designed to improve the fidelity of replication. Typically, rates of mutation are in the range  $10^{-9}$ – $10^{-12}$  errors/base pair replicated,<sup>74–76</sup> owing to the accuracy achieved by polymerases and to postreplicative repair systems.<sup>77</sup> The error rates of many polymerases have been measured in the range  $10^3$ – $10^5$  bases polymerized/error by in vitro assays<sup>78</sup> though the energy differences between correctly and incorrectly base-paired duplexes would predict error rates in the range 1–10 errors/100 bases polymerized (1–3 kcal/mol). Thus, the polymerase must amplify or supplement the available energy for discrimination against incorrect nucleotides to achieve acceptable error rates.

The mechanisms of this amplification and their coordination with the required rapid rates of polymerization have been the subject of intense scrutiny.<sup>79</sup> Many models have been proposed to account for the high fidelity.<sup>80–85</sup> All involve at least two distinct selection steps to increase discrimination. Generally, these two steps are the ability to discriminate against the incorrect nucleotide during binding and polymerization and the subsequent editing of errors by a 3' → 5' exonuclease activity. This section will concentrate on three general methods used for assessing discrimination against incorrect bases, discuss the contribution of 3'-exonuclease to fidelity in the context of its coordination with the polymerase activity, and evaluate the experimental results in terms of models for fidelity previously presented.

#### A. Nonsense Codon Reversion and Forward Mutation Assays

Assays of misincorporation measuring reversion rates or forward mutation rates are based on in vitro completion of a gapped double-strand vector by a given polymerase, as diagrammed in Figure 15. The completed vector is then used to transfect cells or spheroplasts. The presence of a mutation in the portion of the vector that was replicated in vitro is noted as a change in phenotype of infected plaques. The precise nature of the mutation is determined by sequencing DNA from selected mutant plaques. Early work used DNA from phage  $\Phi$ X174 containing amber codons, which generate nonsense stop signals in essential genes<sup>86</sup> or in the *lacZα* complementation gene.<sup>87</sup> The *lacZα* gene encodes the N-terminal portion of  $\beta$ -galactosidase, which produces a blue color in phage plaques on indicator agar plates containing X-gal. A more recent method that allows a wider range of misincorporations uses M13mp2 containing the *lacZα* complementation gene. Mutations at about 100 different sites of *lacZα* have been shown to cause observable changes in phenotype, the change from blue plaques to light blue or colorless plaques.<sup>88</sup> The variety of sites available in this forward mutation assay allow evaluation of all 12 possible misincorporation reactions and in many cases the effects of different neighboring sequences. The frequency of a given misincorporation may be calculated, and results are generally expressed as the number of mutations per bases replicated. This discussion will be limited to isolated polymerases. Work with more complex systems has suggested their fidelity to be higher than that of isolated systems.<sup>98</sup>

##### 1. Eukaryotic Polymerases

Comparative studies using the forward mutation assay of the three major classes of eukaryotic polymerases,  $\alpha$ ,  $\beta$ , and  $\gamma$ , have revealed substantial differences in the frequency and type of mutation created.<sup>89,90</sup> The overall misincorporation frequencies ranged from 1 in 130 bases to less than 1 in 830 000 bases. A number of factors were identified that influenced the error rate. The polymerase used had a profound effect on error rates. In general, the trend of increasing fidelity among the

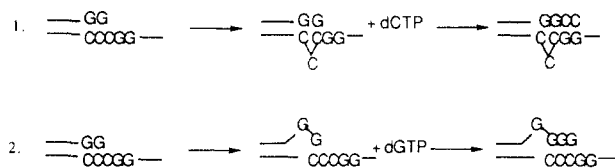


Figure 16. Transient dislocation mechanism.

three polymerases was  $\text{pol } \beta < \text{pol } \alpha < \text{pol } \gamma$ . On average, the overall error rates were 1 in 1300 to 1 in 1500, for  $\text{pol } \beta$ , depending on the source of the enzyme; 1 in 3100 to 1 in 4700, for  $\text{pol } \alpha$ ; and 1 in 6300 to 1 in 12000, for  $\text{pol } \gamma$ . The average overall frequencies of misincorporation for even the least accurate polymerase,  $\text{pol } \beta$ , is still 10–100 times greater discrimination than the energy differences of correctly vs incorrectly based-paired duplex, even in the absence of a 3'-exonuclease that could serve as an editing mechanism.

The frequency of forming a given mismatch varied substantially among the three polymerases. For example, KB cell  $\text{pol } \alpha$  formed a G:T mismatch (in this review all mismatches given as X:Y mean X is being misincorporated opposite a template containing Y) 12-fold less frequently than did  $\text{pol } \beta$ . However, in some cases  $\text{pol } \alpha$  formed mismatches more frequently than did  $\text{pol } \beta$ . The mismatch frequency was not symmetrical in that G:A could be formed 7-fold more frequently than A:G with the same polymerase. Different sequence contexts could cause as much as a 9-fold difference in misincorporation frequency of a given mismatch. The general trend in the frequency of misincorporation opposite any template position was for all polymerases  $A > G > C > T$ , the same order as the trend observed opposite abasic lesions in DNA<sup>91</sup> and the same trend as predicted on the basis of model studies,<sup>92</sup> suggesting the possibility that misincorporations do not use any of the available hydrogen-bonding information. In general, the source of the enzymes used made little difference in the observed mismatch frequencies, suggesting a given class of polymerase uses much the same mode of interaction with its substrates. The correlation between higher fidelity and higher processivity among eukaryotic polymerases was noted.<sup>78</sup>

## 2. Transient Misalignment

More complex mutations can be created during replication by the addition or deletion of bases and can be detected by the forward mutation assay. Frame shift mutations have been proposed to occur through an intermediate containing a dislocation of primer relative to template,<sup>93,94</sup> as illustrated in Figure 16. The temporary misalignment would generate an extrahelical base on either the template or primer strand to generate either a -1 or +1 base frame shift, respectively. The extrahelical base may be located at the primer terminus or at some distance from it. The frequency of frame shifts created during synthesis by human  $\text{pol } \alpha$  and by rat  $\text{pol } \beta$  were shown to have a 10-fold greater amount of -1 frame shifts to +1. The bias against +1 frame shifts has been attributed either to the higher total number of base pairs that must be broken and then re-formed to create a +1 frame shift or to some structural constraint on polymerase binding that preferentially works against an extrahelical primer base.<sup>78</sup> Overall,  $\text{pol } \beta$  produced frame shift mutations about 7-fold more frequently than did  $\text{pol } \alpha$ . Both enzymes

caused frame shift mutations more often at reiterated runs of a single base than at random sequences. Most remarkably,  $\text{pol } \beta$  caused a deletion at one run of four T's in 1 out of 57 bases replicated. The deletion frequency was higher at polypyrimidine runs than at polypurine runs, possibly because of less base overlap energy allowing an easier unstacking.<sup>78</sup> Base dislocation can also lead to error by a mechanism including an initial misalignment, incorporation, and then realignment.

## 3. Reverse Transcriptases

The fidelity of reverse transcriptases (RT) in carrying out DNA-directed DNA synthesis has also been evaluated by these genetic assays.<sup>95,96</sup> RT from the human immunodeficiency virus (HIV) was found to be about 10-fold less accurate than either avian myeloblastosis virus RT or murine leukemia virus RT, supporting the view that the rapid mutational frequency and variety of different strains of HIV are, at least in part, due to the low fidelity of its RT. Overall, HIV RT was found to be of comparable accuracy to  $\text{pol } \beta$ . As with the eukaryotic polymerases, error rates for different mismatches created by HIV RT were shown to be widely variable.<sup>97</sup>

## 4. KF and taq Polymerase

The fidelity of  $\text{pol } I$  in copying  $\Phi X174$  DNA containing an amber mutation has revealed frequencies of mutation for a number of specific mismatches.<sup>99</sup> Misincorporation of C:A occurred at 1 in  $6.8 \times 10^5$  bases, and A:A occurred at less than 1 in  $6.3 \times 10^6$  bases. Misincorporation of G occurred at 1 in  $1 \times 10^6$  bases. By comparison,  $\text{pol } I$  is substantially more accurate than the eukaryotic polymerases and AMV RT but less accurate than either *E. coli*  $\text{pol } III$  or T4 polymerase.<sup>79</sup> A direct comparison of the fidelity of synthesis by KF and the highly homologous polymerase from *Thermus aquaticus* (*taq*) has been determined by the forward mutation assay.<sup>100</sup> The fidelity of *taq* is of special importance because of its widespread use in the polymerase chain reaction (PCR<sup>101</sup>) for amplification of DNA samples in cloning and diagnostics. *taq* was found to be about 4-fold less accurate in DNA synthesis at 55 °C than KF at 37 °C assayed under conditions that suppress the 3' exonuclease activity of KF despite the lack of any detectable 3' exonuclease activity associated with *taq*. Higher assay temperatures, 70 °C, with *taq* led to only a slight increase in mutation frequency.

The use of codon reversion and forward mutation assays allows an examination of the relative frequency of base substitution and frame shift errors. The application of these methods to a wide range of polymerases from different sources has led to increased knowledge of the range of fidelity that occurs during DNA synthesis and some of the factors influencing it. However, detailed investigation of the contribution of the polymerase to the kinetics of the misincorporation reaction, and therefore to the different energetics of correct and incorrect synthesis, requires measurement of reaction rates as described below.

## B. Gel Assay

One approach to measuring the kinetic parameters associated with misincorporation reactions involves the

use of a gel assay for substrate and product separation and steady-state reaction conditions.<sup>102</sup> The substrate is designed to allow two correct incorporations, and then misincorporation is quantitated at the third site. The advantage of this template design is that a direct measurement of reaction velocities can be made by comparison of band intensities of the second and third extended primer positions. Analysis of *Drosophila* pol  $\alpha$  by the gel assay technique revealed a frequency of misinsertion of G opposite template T of  $2 \times 10^{-4}$  and of C opposite T of  $5 \times 10^{-5}$ . These low misinsertion frequencies were attributed to a large 1100-fold increase in  $K_M$  for dGTP relative to dATP and a modest 4-fold decrease in  $V_{\max}$ . Therefore, pol  $\alpha$  was concluded to operate more by a mechanism of  $K_M$  discrimination than by  $V_{\max}$  discrimination.<sup>85</sup>  $K_M$  discrimination should not be interpreted as necessarily meaning discrimination during the binding of dNTP since, in at least some cases,  $K_M$  will not equal  $K_D$ , the thermodynamic binding constant. The application of the gel assay technique to other polymerases should take into consideration the assumptions used in the generation of the kinetic expressions used to describe reaction profiles from the gel assay.<sup>102</sup>

A thorough examination of nearest-neighbor influences on insertion fidelity by means of the gel assay lead to a correlation between  $K_M$  or  $V_{\max}$  discrimination and nearest-neighbor effects.<sup>103</sup> Under  $K_M$  discrimination conditions misinsertion hot spots were found to occur at positions where the 5'-base was a pyrimidine. Under  $V_{\max}$  discrimination conditions greater misinsertion frequency was observed at positions with a 5'-purine.

The transient misalignment hypothesis described in section IV.A was also investigated in the gel assay system.<sup>104</sup> Misalignment mutagenesis was shown to be much more prevalent with pol  $\beta$  than pol  $\alpha$  or yeast DNA pol I. Dislocation errors by pol  $\beta$  were characterized by a lowered  $K_M$  for incorporation relative to the  $K_M$  for a standard direct misinsertion. Comparison to the results from forward mutation assays for dislocation<sup>93</sup> revealed both methods gave an increased frequency of misinsertion at positions where dislocation was possible.

An attempt to reconcile kinetic data regarding 3'-terminal mismatches and thermodynamic data from thermal melting has been made.<sup>105</sup> Free energy differences derived from measurements of the rate of formation and extensions of mismatches were determined to be generally >10 times the free energy differences found from thermodynamic melting studies. One possible cause of the differences found may be exclusion of water from the active site of the polymerase-DNA complex that would serve to increase the difference in energy between correctly and incorrectly base-paired DNA.<sup>106</sup>

### C. Pre-Steady-State Methods

#### 1. Mechanism of Misincorporation

The observation that KF can catalyze the misincorporation of bases into synthetic DNA duplexes has led to a detailed investigation of the kinetics of misincorporation and a general kinetic mechanism governing misincorporation,<sup>107</sup> given in Figure 17. As with correct incorporation, KF associates first with DNA and then with incorrect dNTP. The conformational change to

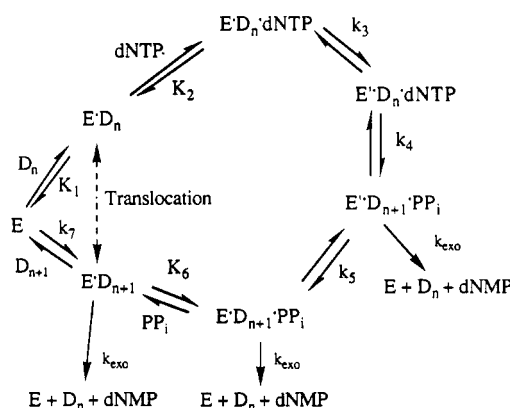


Figure 17. Kinetic mechanism of misincorporation.

the activated complex then follows, and the chemical bond is formed in step  $k_4$ . A second conformational change follows that serves to hold mismatched product on the polymerase for an additional time and is followed by dissociation of products  $PP_i$  and  $DNA_{n+1}$ .

There are two major differences between the mechanism for correct synthesis as discussed in section III.B and that for incorrect synthesis. First, the rate under single-turnover conditions (pre steady state) is now limited by  $k_4$ , the rate of chemical step, instead of  $k_3$ , the rate of conformational change, as for correct synthesis. Single-turnover conditions serve to eliminate product off-rates from contributing to the observed rates, since every substrate molecule is bound by an enzyme molecule. The change in the rate-limiting step is demonstrated by the observation of a large decrease in rate on substituting an  $\alpha$ -thio-dNTP for dNTP.

The second major change is the increased kinetic importance of the second conformational change,  $k_5$ , that follows misincorporation during incorrect synthesis. The effect of the second conformational change is to hold mismatched product DNA onto KF, allowing the 3' exonuclease additional time to remove the mismatch. The evidence for the necessity of a second conformational change is twofold.

First, the steady-state rate of misincorporation is slower than either the rate of the  $k_4$  step measured with an excess of polymerase over substrate DNA or the rate of release of mismatched product DNA. The off-rate of mismatched product DNA was determined by preincubating a preformed mismatch with KF and then adding a standard duplex (13/20 mer) and its next correct dNTP. The lag in the production of 14/20 mer relative to its production in the absence of mismatched DNA is characteristic of the rate of release of the mismatch. Off-rates in the range  $1\text{--}3\text{ s}^{-1}$  for mismatches were measured and can be compared to off-rates of  $0.06\text{--}0.17\text{ s}^{-1}$  for correctly base-paired duplex.

Second, the existence of step  $k_5$  is necessary to explain how some mismatches are able to build under conditions of excess KF and in the presence of 3' exonuclease activity while other mismatches are formed and then excised before being released from KF. For example, an A:A mismatch was formed and built to  $\sim 50\%$  of the available DNA whereas addition of dCTP to 9/20 mer led to the production of dCMP by incorporation and rapid excision but no observable amount of 9C/20 mer was formed.

Quantitative evaluation of the pre-steady-state parameters for misincorporation of dATP opposite a



## Selectivity Obtained by KF at Each Stage

stage 1	$1.1 \times 10^4$ – $1.2 \times 10^6$
stage 2	4–60
stage 3	6–340

**Figure 18.**

template A revealed a decrease in the observed rate of misincorporation relative to correct synthesis of 1500-fold ( $0.03$  vs  $50 \text{ s}^{-1}$ ). The binding constant for the incorrect base as either the measured  $K_M$  of modeled  $K_D$  from computer simulation was  $40 \mu\text{M}$  as compared to  $5 \mu\text{M}$  for the correct base, an 8-fold increase. Therefore, for the A:A mismatch, KF gains most of its discrimination against the incorrect base by means of a decrease in rate of reaction and only a slight increase in the binding constant for the incorrect base.

After the mismatch is formed, KF has a choice of dissociation, exonuclease, or addition of the next correct base onto the mismatch. Measurement of rates of extension of three mismatches revealed a wide range of rates from  $<10^{-5} \text{ s}^{-1}$  for extension of a T:T mismatch to  $10^{-2} \text{ s}^{-1}$  for extension of a C:C mismatch. These rates are much slower than the  $50 \text{ s}^{-1}$  measured for extension of correctly base-paired primer terminus. The slower addition onto a mismatched primer terminus gives KF an additional opportunity to excise the mismatch before it is locked in by extension, an effect that has also been observed for pol  $\alpha$ .<sup>105,108</sup>

Direct comparison of the kinetic mechanisms of correct and incorrect incorporation has led to the division of fidelity into three stages of selection, summarized in Figure 18. Stage 1 selection occurs during dNTP binding and phosphodiester bond formation. Limits on the amount of quantitative selection were estimated by comparing the slowest and fastest measured misincorporation to the rate of correct synthesis, which is limited by the first conformational change, to give a selectivity in the range  $10^4$ – $10^6$  for stage I. The purpose of the first conformational change may be to increase selectively the rate of the chemical step for correct synthesis from some value less than  $50 \text{ s}^{-1}$  to a rate much greater than  $50 \text{ s}^{-1}$ . Stage 2 selection occurs during the second conformational change, the  $k_5$  step, that allows KF additional time to excise mismatches before dissociation of the DNA. Comparison of the ratio of the rates  $k_5$  and  $k_{\text{exo}}$  for correct and incorrect incorporation led to a range of 4–60 for selectivity during stage 2. The final stage of selectivity occurs when KF has the choice of extending a mismatch. The reduced rate of extension onto a mismatch adds another factor of 6–340-fold increase in selectivity. When KF functions optimally, the overall selectivity is predicted to be  $2 \times 10^{10}$ , near the maximum predicted values for DNA synthesis.<sup>74</sup>

## 2. Work in Progress

Current efforts are directed toward extending the mechanism of misincorporation to cover all possible mismatches, to assessing the effects of various mutagenic agents on the rates involved in misincorporation, and to increasing the understanding of the contribution of individual amino acids of KF to the overall fidelity of KF. The availability of KF(exo<sup>-</sup>)<sup>15</sup> that is devoid of

3' exonuclease activity has allowed the evaluation of the contribution of the 3' exonuclease activity to the mechanism. The effects of removing  $k_{\text{exo}}$  from the mechanism of misincorporation are to decrease both internal proofreading on newly synthesized DNA when the DNA is unable to dissociate, that is prior to  $k_5$ , and external proofreading of DNA that has dissociated. The presence of 3' exonuclease increased the accuracy of synthesis about 30-fold.<sup>109</sup> Measurement of the pre-steady-state misincorporation rates for all 12 mismatches using KF(exo<sup>-</sup>) has shown a 1000-fold range in rates and a 60-fold range in  $K_M$ .<sup>110</sup>

Many single amino acid replacements around the putative primer terminus binding site have been made.<sup>111</sup> Evaluation of the misincorporation rates of one of these mutants, Y766S, has shown it has an increased efficiency of misincorporation of T:G mismatches, 44-fold over KF, while catalyzing correct incorporation at about 20% of the efficiency of KF. Therefore, Tyr-766 is important in maintaining the accuracy of KF, and KF actively participates in the reduction of misincorporations during the polymerization event.<sup>110</sup>

The mutagenic effect of  $\text{Mn}^{2+}$  was found to be the result of changes in several rate constants. Use of  $\text{Mn}^{2+}$  increases the rate of misincorporation, decreases internal proofreading, and decreases external proofreading by increasing the rate of addition of the next correct base onto a preexisting mismatch.<sup>109</sup>

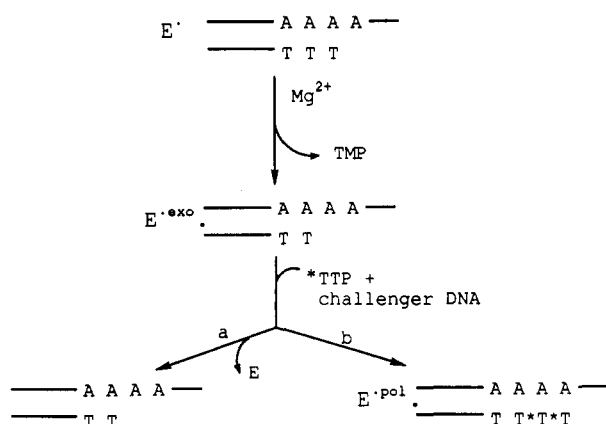
## D. Coordination of 3'-Exonuclease and Polymerase Activities

In order to enhance the fidelity of replication, the 3' exonuclease activity must act in coordination with the polymerase activity. This requirement creates several criteria that must be satisfied for exonucleolytic proofreading, as suggested by Kunkel.<sup>112</sup> The 3' exonuclease should preferentially excise a mismatched primer terminus, be temporally coordinated with the polymerase such that the time available to extend a mispair is minimized (most easily satisfied through a physical association), and act to increase fidelity in some measurable way.

The most convincing evidence for a proofreading exonuclease is found with KF. The 3' exonuclease was found to excise efficiently mismatched bases from the primer terminus.<sup>113</sup> Conditions inhibiting 3' exonuclease were shown to decrease the accuracy of synthesis. High levels of dNMP inhibit exonuclease by binding at the 3' exonuclease active site.<sup>14</sup> High levels of dNTP increase the rate of addition onto a mismatch and so decrease the exonuclease rate, the so-called next-nucleotide effect.<sup>114</sup> The 3' exonuclease is unable to excise  $\alpha$ -thio nucleotides once incorporated into the primer, and their substitution for dNTP decreases fidelity.<sup>115</sup>

The physical separation of the active sites for polymerase and exonuclease activities by some 30 Å creates the problem of the dynamics of switching of the primer terminus from one site to the other, as described in section II. The distance is sufficient that a shifting of the DNA by five bases to the 5'-side of the primer terminus and a melting of four base pairs would be necessary to bridge the gap, as is suggested to occur in the slide and melt hypothesis.<sup>16</sup> Whether the translo-





**Figure 19.** Experimental protocol for inter- vs intramolecular switching from polymerase to exonuclease site.

cation of the DNA could occur without dissociation of the DNA was investigated by carrying out the exonuclease reaction on a primer terminus in the presence of an excess of trapping DNA sufficient to prevent re-binding of the substrate DNA once it had dissociated,<sup>116</sup> as illustrated in Figure 19. The 3'-terminal base could be excised, suggesting an intramolecular switch from polymerase to exonuclease active site. More extensive investigations using the same sort of experimental protocol with a ddNMP-terminated primer have revealed that though intramolecular transfer of product DNA can occur with correctly base-paired DNA, with a 3'-terminal mismatch the preferred mode of transfer is by way of dissociation and then re-binding at the exonuclease active site.<sup>117</sup> The results from trapping experiments are in agreement with the rate constants determined by pre-steady-state methods since the measured off-rate for mismatched DNA is much faster than the 3' exonuclease rate ( $1-3 \text{ s}^{-1}$  vs  $10^{-2} \text{ s}^{-1}$ ). The choice of inter- vs intramolecular proofreading should vary for different mismatches depending on the different values for  $k_{\text{cat}}$ ,  $k_5$ , and the dissociation rate as discussed in section IVC. Dissociation of the enzyme from a mismatch would also allow any other exonuclease present in the cell access to remove the mistake.

One of the suggested criteria for establishing exonucleolytic proofreading is that the activity should be specific for mismatched vs correctly base-paired primer termini. This specificity need not be a property of the exonuclease activity alone, however. The exonuclease activity may be preferentially directed at mismatches by the slow addition of the next correct base onto the mismatch. For example, KF has a high  $k_{\text{exo}}$  on mismatched primer termini but also has a higher  $K_M$  for mismatched DNA,<sup>107</sup> such that the specificity,  $k_{\text{exo}}/K_M$ , does not vary substantially for correctly vs incorrectly base-paired duplexes. However, due to the second conformational change,  $k_5$  step, and to the slow addition of the next correct base onto mismatches, the exonuclease activity is preferentially expressed on mismatches.

The participation of proofreading exonucleases in synthesis by other polymerases has also been demonstrated. The classic studies<sup>118,119</sup> of mutator and antimutator alleles of the T4 polymerase gene have shown the correlation between decreased pol to exo rate ratio and decreased rate of mutations.<sup>120</sup> The  $\epsilon$  subunit of

the multisubunit pol III, the major replicative polymerase in *E. coli*, contains a 3' exonuclease activity and shows a slight homology with the exonuclease domain of KF. Mutants of the  $\epsilon$  gene with decreased 3' exonuclease activity have increased rates of mutation,<sup>121</sup> so  $\epsilon$  may serve a proofreading capacity. The suggestion has been made that the fidelity of synthesis by pol III may be regulated in vivo by the relative levels of the polymerase and  $\epsilon$  subunits.<sup>122</sup> Some eukaryotic polymerases have associated 3' exonucleases. Pol  $\alpha$ , thought to be the major replicative polymerase in eukaryotes, is usually isolated without an associated exonuclease. However, pol  $\alpha$  isolated from several sources<sup>123-125</sup> including HeLa cells, calf thymus, or *Drosophila* has exonuclease activity. Pol  $\beta$ , the eukaryotic polymerase thought to act mainly in repair, is usually isolated without exonuclease activity though it can associate with DNase V, which contains both 3' and 5' exonuclease activities.<sup>126</sup> The mitochondrial polymerase, pol  $\gamma$ , from chick embryo has been shown to contain 3' exonuclease activity that functions in proofreading.<sup>127</sup> Pol  $\delta$ , from bone marrow and calf thymus, has an associated 3' exonuclease activity that can function in a proofreading capacity.<sup>128</sup>

## V. Conclusion

Whether the X-ray crystallographic studies of other polymerases will reveal structures with similar DNA binding clefts and spatially separate polymerase and exonuclease sites is debatable; however, primary sequence homologies in key regions especially for T7 and pol I favor this possibility. In the absence of such data, the use of fluorescent nucleotides and oligonucleotide donor-acceptor pairs should provide information on cavity size and site separation. Biotinylated DNA duplexes in combination with duplexes containing intrastrand chemical cross-links also can be used to determine site separation and the requirement for strand melting to access the exonuclease site.

Similarly, a commonality in kinetic sequence probably exists for the various polymerases that features ordered DNA followed by dNTP binding, a rapid chemical step flanked by ternary complex conformational changes, and slow duplex product release. The slowness of this DNA release step relative to initiation of a new round of synthesis acts to set the degree of polymerase processivity. In order to achieve high fidelity of polymerization, different steps within this kinetic sequence will be used to discriminate a correct from an incorrect base depending on the source of the enzyme. One can expect discrimination to be achieved in three key steps: dNTP binding, the rate of chemical polymerization, and the rate of ternary product complex transit relative to the exonuclease activity. All three will vary with the identity of the enzyme. The contributions as well as the numbers of individual amino acids within the polymerase active sites that ensure high fidelity of synthesis have not yet been elucidated, and it is this further reduction of the problem to molecular events that is eagerly awaited.

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**Registry No.** DNA polymerase, 9012-90-2.

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