Mechanism of DNA Replication Fidelity for Three Mutants of DNA Polymerase I: Klenow Fragment KF(exo$^+$), KF(polA5), and KF(exo$^-$)†

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ABSTRACT: Inhibition of the pre-steady-state burst of nucleotide incorporation by a single incorrect nucleotide (nucleotide discrimination) was measured with the Klenow fragment of DNA polymerase I [KF(exo$^+$)]. For the eight mispairs studied on three DNA sequences, only low levels of discrimination ranging from none to 23-fold were found. The kinetics of dNTP incorporation into the 9/20-mer at low nucleotide concentrations was also determined. A limit of ≥ 250 s^{-1} was placed on the nucleotide off-rate from the KF(exo$^+$)–9/20–dTTP complex in accord with nucleotide binding being at equilibrium in the overall kinetic sequence. The influence of the relatively short length of the 9/20-mer on the mechanism of DNA replication fidelity was determined by remeasuring important kinetic parameters on a 30/M13-mer with high homology to the 9/20-mer. Pre-steady-state data on the nucleotide turnover rates, the dATP(αS) elemental effect, and the burst of dAMP misincorporation into the 30/M13-mer demonstrated that the kinetics were not affected by the length of the DNA primer/template. The effects on fidelity of two site-specific mutations, KF(polA5) and KF(exo$^-$), were also examined. KF(polA5) showed an increased rate of DNA dissociation and a decreased rate of polymerization resulting in less processive DNA synthesis. Nevertheless, with at least one misincorporation event, that of dAMP into the 9/20-mer, KF(polA5) displays an increased replication fidelity. By comparison of KF(exo$^-$) to KF(exo$^+$) the exonuclease activity was shown to enhance fidelity by as much as a factor of 30 through contributions to the stages of both internal proofreading (i.e., a stage where the newly elongated enzyme–DNA$_{n+1}$ complex was unable to dissociate) and external proofreading (i.e., a stage where the DNA$_{n+1}$ freely dissociated from the enzyme–DNA$_{n+1}$ complex). The KF(exo$^-$) was also used to demonstrate that Mn^{2+} decreases fidelity at all three stages by (i) increasing the net rate of insertion of the incorrect nucleotide, (ii) decreasing the level of internal proofreading, and (iii) decreasing the level of external proofreading.

DNA polymerase I (pol I)[1] from *Escherichia coli* is a monomeric enzyme involved with DNA-dependent DNA repair synthesis (Kornberg, 1980). The enzyme contains separate active sites for the catalysis of DNA polymerization and of hydrolysis from either the 3′- or 5′-ends. Important features of the reaction mechanism for DNA replication have been elucidated through structural (Ollis et al., 1985; Joyce & Steitz, 1987; Freemont et al., 1988; Cowart et al., 1989; Catalano & Benkovic, 1989; Catalano et al., 1990), stereochemical (Burgers & Eckstein, 1979; Brody & Frey, 1981; Gupta & Benkovic, 1984), and kinetic studies (Travaglini et al., 1975; McClure & Jovin, 1975; Bambara et al., 1976; Bryant et al., 1983; Mizrahi et al., 1985, 1986; Kuchta et al., 1987, 1988). Removal of the domain containing the 5′→3′ exonuclease active site either by limited proteolysis (Brutlag et al., 1969; Klenow & Henningsen, 1970) or by genetic means (Joyce & Grindley, 1983) generates an enzyme designated as the Klenow fragment [KF(exo$^+$)] which still contains full polymerase and 3′→5′ exonuclease activities.

Using the Klenow fragment, we recently determined the minimal kinetic schemes that accurately describe correct and incorrect polymerization (Kuchta et al., 1987, 1988). The quantitative differences between the mechanisms for correct and incorrect synthesis may be divided into three stages that contribute to overall replication fidelity (Figure 1). The three stages are (1) the increased free energy barrier associated with phosphodiester bond formation for misincorporation, (2) a slower rate for the conformational change following phosphodiester bond formation that increases the residence time of incorrect DNA products, thereby allowing the slow 3′→5′ exonuclease activity additional time to remove incorrect bases before dissociation of the DNA (internal proofreading), and (3) the reduced rate of addition of the next correct nucleotide onto a mismatch, again allowing the exonuclease longer time to remove incorrect bases (external proofreading). Most replication fidelity is achieved in the first stage through differences in the rate of correct vs incorrect nucleotide insertion (Kuchta et al., 1988).

The present study seeks to assess the validity of the proposed mechanism of misincorporation (Kuchta et al., 1988) and to interpret observations from previous steady-state experiments in terms of the overall mechanism for misincorporation. The inhibition of incorporation of a correct nucleotide by the

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[1] Abbreviations: pol I, DNA polymerase I; KF and KF(exo$^+$), the Klenow fragment of DNA polymerase I; KF(exo$^-$), a site-specific mutant (Asp355Ala, Glu357Ala) of the Klenow fragment with diminished 3′→5′ exonuclease activity; KF(polA5), a mutant (Gly850Arg) of the Klenow fragment; EDTA, ethylenediaminetetraacetate, sodium salt; Tris-HCl, tris(hydroxymethyl)aminomethane, hydrochloride salt; TEAB, triethylammonium bicarbonate.

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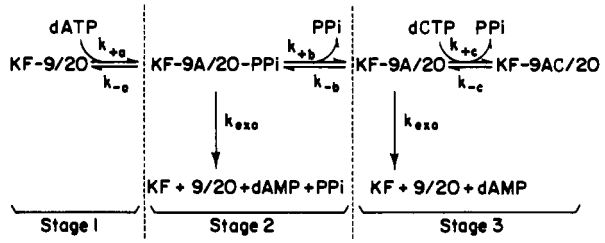


FIGURE 1: Three stages of stable incorporation of a nucleotide (correct or incorrect) which contribute to low levels of misincorporation. The stages represent (1) nucleotide discrimination and incorporation, (2) partitioning between pyrophosphate release and exonucleolytic cleavage, and (3) nucleotide addition onto the 3′-primer terminus (matched or mismatched). The rate constants for each stage have been determined by computer simulations (KINSIM) to a single mechanism which fits all of the data for dATP misincorporation (Kuchta et al., 1988): $k_{+a} = 770\ M^{-1}\ s^{-1}$; $k_{-a} = 3.3 \times 10^{-3}\ s^{-1}$; $k_{+b} = 3.5 \times 10^{-3}\ s^{-1}$; $k_{-b} = 35\ M^{-1}\ s^{-1}$; $k_{exo} = 0.009\ s^{-1}$; k_{+c} and k_{-c} have not been uniquely determined. However, the k_{cat} for addition of the correct nucleotide (dCTP) onto the 9A/20-mer has been shown to be $< 2 \times 10^{-5}\ s^{-1}$.

presence of an incorrect nucleotide was determined by an independent measurement of the binding of the incorrect nucleotide. The generality of the scheme for nucleotide misincorporation was tested by examining misincorporation with an M13 primer/template system. In addition, the effects on the kinetic mechanism of two site-specific mutants, KF(polA5) and KF(exo$^-$), were determined, the latter being used to assess the contribution of the exonuclease activity in enhancing fidelity. Finally, the changes in misincorporation rates caused by substitution of the mutagenic metal cofactor Mn^{2+}, for Mg^{2+}, were also measured.

Experimental Procedures

Materials

Radioactive nucleotides were from NEN. Unlabeled dNTPs were from Pharmacia. (S_P)-dATP(αS) was synthesized and purified by published procedures (Chen et al., 1983). The KF(exo$^-$) (Derbyshire et al., 1988) and KF(exo$^+$) (Joyce & Grindley, 1983) were purified as described previously. To obtain maximal purity, KF(exo$^-$) was subjected to chromatography on both Bio-Rex 70 and Sephadex G-150. Protein concentrations were assessed with $\epsilon_{278} = 6.32 \times 10^4\ M^{-1}\ cm^{-1}$ (Setlow et al., 1972). The KF(polA5) was purified from an *Escherichia coli* overproducer. Except for the lysozyme treatment, all operations were performed at 4 °C. Cells (4 g) were lysed by treatment with lysozyme (20 mg mL^{-1}) for 30 min at 22 °C followed by sonication, and cell debris was removed by centrifugation for 15 min at 15000*g*. The supernatant was applied to a 150-mL DE52 (Whatman) column in 10 mM KP_i, pH 7. Proteins were eluted with a 1-L gradients of 0–1 M KCl. Fractions containing activity were pooled, dialyzed against 10 mM KP_i, pH 7, and loaded onto a 30-mL Bio-Rex 70 column equilibrated with this buffer. Proteins were eluted with a 300-mL gradient of 0–1 M KCl in 10 mM KP_i, pH 7. Fractions containing KF(polA5) were pooled. $(NH_4)_2SO_4$ (0.56 mg mL^{-1}) was added, and precipitated proteins were removed via centrifugation. The pellet was resuspended in a small volume of 10 mM KP_i, pH 7, and 1 mM dithiothreitol and then dialyzed against 1 L of 10 mM Tris-HCl, pH 7.4. The enzyme was concentrated by dialysis against 1 L of 10 mM KP_i, pH 7, 1 mM dithiothreitol, and 50% glycerol. The purified KF(polA5) was stored at −70 °C. The protein was >95% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. DNA oligomers were synthesized on an Applied Biosystems 380A synthesizer.

Chart I: Duplex DNA Sequences[a]

DNA Name	Sequence
9/20-mer	TCGCAGCCG(3') AGCGTCGGCAGGTTCCCAAA
10/20-mer	TCGCAGCCGT(3') AGCGTCGGCAGGTTCCCAAA
13/20-mer	TCGCAGCCGTCCA(3') AGCGTCGGCAGGTTCCCAAA
13/20'-mer	TCGCAGCCGTCCA(3') AGCGTCGGCAGGTACCCAAA
14/20-mer	TCGCAGCCGTCCAA(3') AGCGTCGGCAGGTTCCCAAA

[a] The names of each DNA define the length of the oligonucleotides used for the primer/template. DNA sequences that contain additional bases (correct or incorrect) on the 3′-end of the primer contain the identity of those bases in the DNA name (e.g., 9A/20-mer is the 9-mer primer with an additional A on the 3′-end creating an A–A mismatch).

The DNA was purified either by gel electrophoresis or with an Applied Biosystems oligonuceotide purification cartridge. The manufacturer's protocols were used. DNA duplexes (Chart I) were formed and the concentrations determined as previously described (Kuchta et al., 1988). M13mp18(+)-strand DNA was purified by published procedures and quantitated by UV spectroscopy (Johnson, 1987).

Methods

Scintillation counting, denaturing polyacrylamide gel electrophoresis, thin-layer chromatography, and DE81 filter binding assays were performed as described previously (Kuchta et al., 1987, 1988). All operations were performed at room temperature unless noted.

Computer Simulations. Computer simulations were performed with the program SIMUL (Barshop et al., 1983) that was modified to allow input of data as *x,y* pairs (Anderson et al., 1987). Best fits to the data sets (pre-steady-state misincorporation, pre-steady-state incorporation, steady-state misincorporation, and dATP hydrolysis to dAMP) were estimated by visual inspection.

Discrimination during dNTP Binding. DNA duplexes are shown in Chart I. The pre-steady-state incorporation for a single correct nucleotide was measured by rapid quench at 20.0 ± 0.1 °C over a range of reaction times from 0.004 to 10 s. The K_M for the correct nucleotide was calculated from the SIMUL fits of the burst rate as a function of nucleotide concentration over a range near the measured K_M value. Measurements of K_I values were run for individual incorrect nucleotides with concentrations as high as 80 μM in the presence of the correct nucleotide. The K_I was found to be unchanged over the range of inhibitor concentrations, and the inhibition was determined to be competitive.

Length Effects. The DNA sequence of M13, obtained from Genbank (Genbank, 1987), was modified to match the published M13mp18(+)-strand sequence (Yanisch-Perron et al., 1985). In-house computer programs were used to locate the region of highest homology to the 3′-terminus of the 9/20-mer (Chart II). The region of M13mp18 DNA with the next highest homology contained only 19 correct base pairs on the 30-mer sequence. The 30-mer of sequence 5′-d(GGTGCACTAACAACTAATAGATTAGAGCCG), which was complementary to positions 4719–4748 of the M13mp18

Scheme I

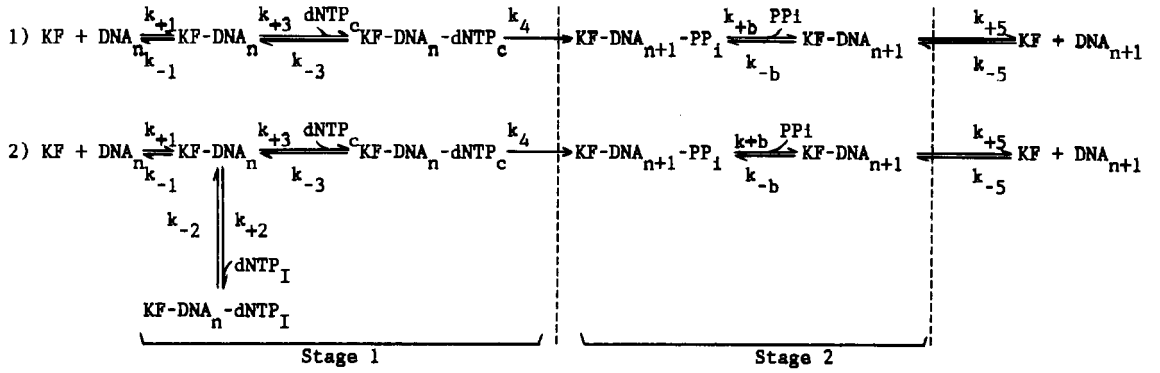


Chart II: Sequence Homologies[a]

GGTGCACTAACAACTAATAGATTAG**AGCCG**(3')
(I) M13mp18—CCACGTGATTGTTGATTATCTAATC**TCGGCAG**TTA—

TCGC**AGCCG**(3')
(II) AGCG**TCGGCAG**GTTCCCAAA

[a] DNA sequences of the 30/M13-mer around the primer binding site (sequence I) and the 9/20-mer (sequence II) with the homologous bases in boldface type.

DNA, was synthesized and purified.

DNA hybridization mixes contained 124 nM M13mp18 and 87 nM 5'-^{32}P end-labeled 30-mer with the pre-steady-state misincorporation assays containing 35 nM 30/M13-mer and 700 nM either KF(exo$^+$) or KF(exo$^-$). Control experiments demonstrated that a 20-fold excess of either KF(exo$^+$) or KF(exo$^-$) was required to saturate the primer 3'-terminus (data not shown). Therefore, all of the experiments were run under pre-steady-state conditions with a 20-fold excess of enzyme over primer terminus. Other experimental protocols used were as previously documented (Kuchta et al., 1987, 1988).

Kinetic Parameters of KF(polA5). Misincorporation of dATP into 9/20-mer, exonuclease activity (k_{exo}), and pre-steady-state polymerization (k_4 in Scheme I) as catalyzed by KF(polA5) were determined as described previously (Kuchta et al., 1987, 1988). To measure the K_D for 13/20-mer, a solution of 20 nM KF(polA5), 15–125 nM 5'-^{32}P-labeled 13/20-mer, and 5 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.4, was incubated for 30 s. Heat-denatured calf thymus DNA (Sigma) and dATP were added to give final concentrations of 2.5 mg mL^{-1} and 32 μM, respectively. Aliquots were removed after a further 20–60 s and quenched with gel loading buffer (90% formamide). The dATP concentration was sufficient to allow conversion of all of the KF(polA5)–13/20-mer to KF(polA5)–14/20-mer. Control experiments, which were run by incubating 20 nM KF(polA5) with 2.5 mg mL^{-1} calf thymus DNA before addition of 5'-^{32}P-labeled 13/20-mer and 32 μM dATP, resulted in a >98% reduction in 14/20-mer formation. After gel electrophoresis and autoradiography, the bands corresponding to 13-mer and 14-mer were excised from the gel and quantified by scintillation counting.

The dissociation rate of 13/20-mer from KF(polA5) was determined by mixing a solution (35 μL) of 100 nM KF(polA5), 100 nM 5'-^{32}P-labeled 13/20-mer, and 1.2 mM EDTA in 50 mM Tris-HCl, pH 7.4, with a solution of calf thymus DNA (6 mg mL^{-1}) and 1.2 mM EDTA in 50 mM Tris-HCl, pH 7.4. After incubation times ranging from 0.01 to 3 s, the reaction solution was mixed with 173 μL of 200 μM dATP and 16 mM $MgCl_2$. Following an additional incubation of 15 s, 70-μL aliquots were mixed with 8 μL of 3 M NaOAc, pH 5, and 195 μL of EtOH and precipitated. The amount of 13-mer and 14-mer was quantified by gel electrophoresis as described above.

Kinetic Parameters for KF(exo$^-$). The rates of DNA dissociation, addition onto a mismatch, pre-steady-state misincorporation, and correct incorporation were determined as described previously (Kuchta et al., 1987, 1988). Steady-state misincorporation of dATP into 9/20-mer was measured in assays (15 μL) containing 2.5–30 μM [α-^{32}P]dATP (approximately 90 000 cpm pmol^{-1}), 1.36 μM 9/20-mer, 33 nM KF, and 5 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.4. After 5 and 25 min, aliquots were quenched and products quantitated by denaturing polyacrylamide gel. The values of K_M(dATP) and k_{cat} were determined as described above. Control experiments had demonstrated that misincorporation was linear for at least 30 min under these conditions.

Mn^{2+}-Induced Mutagenesis. Experimental protocols were identical with those used for $MgCl_2$, except the $MgCl_2$ was replaced with 0.15 mM $MnCl_2$. Control experiments (data not shown) demonstrated this to be the optimal Mn^{2+} concentration. Reaction conditions were as described in this work or previously (Kuchta et al., 1987, 1988).

Results and Discussion

Discrimination during dNTP Binding. Tighter binding of the correct nucleotide relative to the incorrect nucleotides (nucleotide discrimination) (Hopfield, 1974; Ninio, 1975) and higher rates for steps leading to correct incorporation are two mechanisms by which DNA polymerases may enhance replication fidelity. An initial study using the KF(exo$^+$)–9/20-mer complex had shown that the apparent affinities under pre-steady-state conditions for the correct nucleotide (dTTP) or incorrect nucleotide (dATP) were about the same (Kuchta et al., 1988).

Scheme I contains an outline of the experimental design used to measure the level of nucleotide discrimination. The important rate constants for single-nucleotide incorporation are shown in mechanism 1 of Scheme I, which has an expanded stage 1 (Figure 1). The mechanism consists of Klenow binding to the DNA_n and to the correct nucleotide in an ordered sequence followed by nucleotide incorporation, loss of pyrophosphate, and dissociation of product DNA_{n+1} (Kuchta et al., 1987). All of the experiments were run by preforming a kinetically competent KF–DNA_n complex and initiating with Mg^{2+} and nucleotide, thus eliminating the DNA binding step from the kinetic analysis of the product formation. In addition, the exonuclease step is kinetically insignificant on the time scale of a single correct nucleotide incorporation and thus was also eliminated from the kinetic analysis.

The pre-steady-state burst for the incorporation of a single correct nucleotide was measured with a 3-fold excess of DNA_n (Figure 2). The amplitude of the rapid phase in Figure 2 which represents the formation of 1 enzyme equiv of 10/20-mer reflects the conversion of the initial substrate complex to

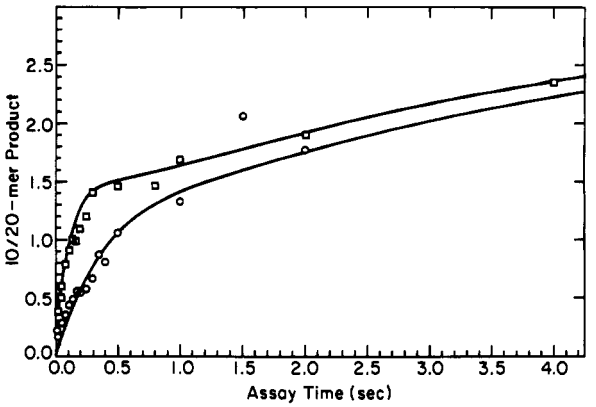


FIGURE 2: Effect of dATP on the pre-steady-state burst of dTTP incorporation into the 9/20-mer to form 10/20-mer. Assays were performed with excess 9/20-mer as described under Experimental Procedures with 5 μM dTTP and either 0 μM dATP (□) or 72 μM dATP (○). The line for dATP = 0 μM was generated by computer simulation using mechanism 1 of Scheme I and the following individual rate constants: $k_{+1} = 1.2 \times 10^7$ M^{-1} s^{-1}; $k_{-1} = 0.06$ s^{-1}; $k_{+3} \geq 1.25 \times 10^7$ M^{-1} s^{-1}; $k_{-3} \geq 250$ s^{-1}; $k_4 = 50$ s^{-1}. The line for dATP = 72 μM was obtained with mechanism 2 of Scheme I by holding the above rate constants fixed and varying the ratio of k_{+2} to k_{-2} with dATP binding kept at equilibrium relative to turnover.

product. The rate of the second phase is limited by the dissociation of the DNA_{n+1} from the binary product complex (k_{+5}). The K_M for each correct nucleotide was obtained from the nucleotide concentration dependence of the rate determined from the rapid phase. Previous studies (Bryant et al., 1983) as well as data presented here to measure the off-rate of the correct nucleotide (dTTP) from the ternary complex [KF(exo$^+$)–9/20–dTTP] have demonstrated that the K_M value [$(k_4 + k_{-3})/k_{+3}$] is equal to the thermodynamic binding constant (K_D) (since $k_{-3} > k_4$, $K_M = k_{-3}/k_{+3} = K_D$).

Steady-state experiments on homopolymers (Travaglini et al., 1975) have demonstrated that the incorrect nucleotide may be a competitive inhibitor of correct nucleotide binding to the enzyme–DNA_n species. In our experiment, addition of the incorrect nucleotide results in an attenuation of the pre-steady-state burst of correct nucleotide incorporation (Figure 2). Previous experiments had shown that no significant misincorporation will occur in the time necessary for a single correct incorporation (Kuchta et al., 1988).

Mechanism 2 of Scheme I outlines the competition between the incorrect and the correct nucleotide for binding to the KF–DNA_n species. The dissociation constant for the incorrect nucleotide ($K_I = k_{-2}/k_{+2}$) was calculated by using the rate constants determined in the absence of $dNTP_I$ (mechanism 1 of Scheme I) and varying the K_I value. The levels of nucleotide discrimination (Table I) were calculated from eq 1.

$$K_I/K_D = (k_{-2}/k_{+2})/(k_{-3}/k_{+3}) \quad (1)$$

These discrimination values were converted to the change in enthalpy ($\Delta\Delta H°$) with eq 2, where $\Delta H°_C$ and $\Delta H°_I$ represent the change in enthalpy for the binding of the correct and incorrect nucleotides.

$$\Delta\Delta H° = \Delta H°_C - \Delta H°_I = RT \ln (K_I/K_D) \quad (2)$$

The measured binding discrimination (K_I/K_D) was in a range of only 0.0–23-fold, (Table I), much lower than the 10^4–10^5-fold range previously predicted from base-pairing energies of rare tautomer forms in the absence of enzyme (Englisch et al., 1985). However, this range of binding energy discriminations of −0.42 to 1.9 kcal/mol was similar to the range of 1.3–4.8 kcal/mol measured for pol I under steady-state conditions with homopolymers (Travaglini et al., 1975). These discrimination results are also similar to those obtained

Table I: Discrimination during Nucleotide Binding by Klenow Fragment

DNA name[a]	K_D for correct nucleotide (μM)	K_I for incorrect nucleotide (μM)	K_I/K_D	$\Delta\Delta H°$ (kcal/mol)
9/20-mer	20 (dTTP)	20 (dATP)	1.0	0.0
-CG		13 (dGTP)	0.63	−0.28
-GCA-		10 (dCTP)	0.50	−0.42
13/20-mer	4.0 (dATP)	93 (dGTP)	23	1.9
-CA		80 (dTTP)	20	1.8
-GTT-				
13/20′-mer	11 (dTTP)	36 (dATP)	3.3	0.72
-CA		180 (dGTP)	16	1.7
-GTA-		32 (dCTP)	2.9	0.64

[a] The bases near the 3′-terminus of each primer/template are included below the DNA name.

from steady-state replication by Klenow on a poly(dT)–oligo(dA) hook polymer that showed only a 6-fold increase in the K_M for dGTP binding (El-Diery et al., 1988).

The levels of nucleotide discrimination were affected by sequence differences in the DNA surrounding the incorporation site. For example, the 9/20-mer and the 13/20′-mer, both of which incorporate dTTP but differ in their local sequences (Chart I), show large differences in the free energy of discrimination for dGTP and dCTP binding of 2.0 and 1.1 kcal/mol, respectively.

Recent studies have demonstrated that *Drosophila melanogaster* DNA polymerase α achieves DNA replication fidelity in part through high levels of nucleotide discrimination. It was also shown that there was a good correlation between relative levels of nucleotide discrimination and the experimentally measured melting temperatures of the mismatched DNA (Petruska et al., 1988). However, published studies of experimentally determined T_Ms containing either a single internal mismatch (Werntges et al., 1986; Aboul-ela et al., 1985) or a mismatch formed at the end of RNA duplexes (Sugimoto et al., 1987) have shown that the T_M values are sensitive to the DNA sequence near the mismatch. At present, T_M values are not available for our DNA sequences.

Recently, a gel assay was described and applied to DNA polymerase α in which incorporation of the incorrect nucleotides relative to the correct nucleotide was expressed in terms of V_{max} and K_M obtained from the dependence of the reaction velocity on the incorporation of both a single nucleotide (correct or incorrect) at the target DNA site and a correct nucleotide at the preceeding site (Boosalis et al., 1987). The assay was run separately for each nucleotide (correct or incorrect) with excess DNA and the product formation velocities determined after many cycles of nucleotide incorporation and product DNA dissociation. Since this method allows for the rapid screening of discrimination, we examined whether it could be applied to Klenow discrimination. For this ratio to be meaningful, as a measure of "K_M" nucleotide discrimination, the validity of a key assumption is necessary, namely, that the rates of incorporation of both the correct and incorrect nucleotides have the same steady-state rate-limiting step under processive conditions as well as gel assay conditions which limits incorporation to two nucleotides. However, for pol I the steady-state rate for correct incorporation using the gel assay reflects the DNA off-rate (Kuchta et al., 1987), and the steady-state rate of misincorporation of dATP into 9/20-mer is limited by the chemical step (Kuchta et al., 1988). In the case of the 9/20-mer the KINSIM-modeled K_M by this gel assay for dTTP is 5 nM, and k_{cat} would be the off-rate of 10/20-mer (0.06 s^{-1}). For the misincorporation reaction of dATP the

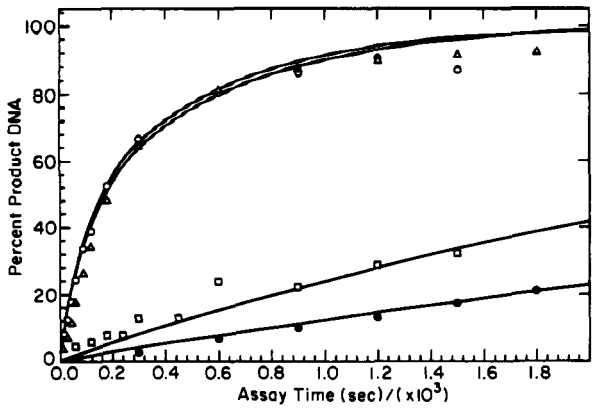


FIGURE 3: Elemental effect on pre-steady-state misincorporation of dATP using KF(exo⁻). Assays were performed with excess enzyme as described under Experimental Procedures. The reaction contained either 7.5 μM dATP (○) or 7.5 μM dATP(αS) (□) with the 9/20-mer and either 20 μM dATP (△) or 20 μM dATP(αS) (●) with the 30/M13-mer. The data for the 9/20-mer with dATP was fit by computer simulation (KINSIM) using the first two stages of the mechanism in Figure 1 and the rate constants in the legend. The data for the 30/M13-mer with dATP were also fit to the mechanism in Figure 1 with $k_{+a} = 270\ M^{-1}\ s^{-1}$ and $k_{-a} = 3.3 \times 10^{-3}\ s^{-1}$ and the other rates from the legend. For dATP(αS) the data for the 9/20-mer and the 30/M13-mer were fit by dividing k_{+a} and k_{-a} by 20 and 40, respectively.

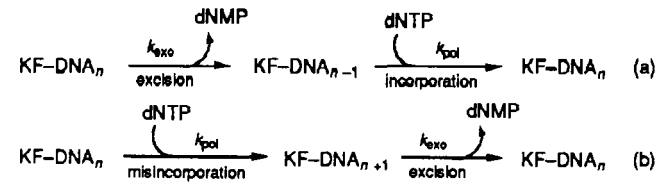
steady-state K_M would be 8 μM, and k_{cat} would be 0.021 s^{-1} (Kuchta et al., 1988). For this mispair the fidelity then would be apparently reflected in K_M which increases by 550-fold as opposed to k_{cat} which decreases by only 3-fold. However, our direct binding measurements on the 9/20-mer substrate have shown that the Klenow fragment binds this incorrect nucleotide as tightly as the correct nucleotide (Table I).

Nucleotide Off-Rate. In this experiment we attempted to separate K_D into its component rates of nucleotide binding (k_{+3}) and nucleotide release (k_{-3}) (Scheme I). At saturating dTTP concentrations the pre-steady-state rate of 10/20-mer formation is fit to a single exponential (Bryant et al., 1983). At lower concentrations, the product formation will become biphasic if the binding step becomes kinetically significant. At dTTP concentrations as low as 0.1 μM only monophasic production of 10/20-mer was observed. Simulations involving steps k_{+3}, k_{-3}, and k_4 in stage 1 showed biphasic appearance of product as a function of time would be detectable when $k_{-3} \leq 250\ s^{-1}$. The value of k_{+3}, set by $K_D = 20$ μM, therefore is greater than or equal to $1.25 \times 10^7\ M^{-1}\ s^{-1}$, within the range of diffusion constants observed (10^6–$10^8\ M^{-1}\ s^{-1}$) for enzyme–substrate association (Fersht, 1985). The binding of dNTP is consequently at equilibrium relative to turnover.

Length Effects. A persistent concern when the DNA duplexes shown in Chart I are being used has been whether the discrimination and overall kinetic sequence determined accurately represents that for longer DNA species found in vivo. To allay this concern, we prepared the 30/M13-mer shown in Chart II. This substrate consists of a circular M13mp18-(+)-strand DNA template primed with a 30-mer, thus generating a more natural primer/template system. The 3′-hydroxyl end of the 30/M13-mer was highly homologous to the 9/20-mer, with the terminal five base pairs in the duplex region and three of the first four single-stranded template bases being identical with the 9/20-mer (Chart II). If the short DNA duplexes are representative of the in vivo substrates, the kinetics of the 9/20-mer previously published (Kuchta et al., 1988) and the 30/M13-mer should be identical.

Misincorporation of dATP into 30/M13-mer using KF(exo⁻) is similar to that of the 9/20-mer in both magnitude and rate of misincorporation (Figure 3). Replacement of dATP with (S_P)-dATP(αS) using KF(exo⁻) resulted in an elemental effect of 40 (30/M13-mer), similar to the elemental effect of 20 observed with the 9/20-mer (Figure 3). This demonstrates that phosphodiester bond formation was at least partially rate limiting for both substrates.

Scheme II



We also wished to examine whether the exonuclease kinetics on the relatively short 9/20-mer were affected by possible loss of crucial enzyme–DNA contacts at the DNA blunt end, since the separation between polymerase and 3′→5′ exonuclease active sites has been estimated as some 30 Å (Ollis et al., 1985) or nine base pairs (Cowart et al., 1989). A comparison of incorrect nucleotide turnover via a nucleotide incorporation/hydrolysis cycle would reflect potential changes in both rates. Scheme II shows two possible mechanisms for a single cycle of nucleotide turnover (Kuchta et al., 1988). The rates of nucleotide removal or insertion (correct or incorrect nucleotide incorporation) are designated k_{exo} or k_{pol}. The net rate (k_{net}) for each cycle of turnover for either mechanism may be calculated from eq 3 (Cleland, 1975).

$$k_{net} = \frac{1}{1/(k_{exo}) + 1/(k_{pol})} \qquad (3)$$

Under the experimental conditions of dNTP = 5 μM, the excision/incorporation pathway shown in eq a of Scheme II (Kuchta et al., 1988) gives $k_{net} = k_{exo}$ since $k_{pol} \gg k_{exo}$. This mechanism predominates for dGTP turnover on the 9/20-mer (Kuchta et al., 1988). Gratifyingly, the nucleotide turnover number of $5.6 \times 10^{-4}\ s^{-1}$ for dGTP on the 30/M13-mer is similar to the value of $3.0 \times 10^{-4}\ s^{-1}$ measured on the 9/20-mer, indicating that the 3′→5′ exonuclease activity has not changed. On the other hand the turnover of dATP on the 9/20-mer follows eq b of Scheme II (Kuchta et al., 1988). The value of $1.3 \times 10^{-3}\ s^{-1}$ measured for the 9/20-mer compares favorably with $1.0 \times 10^{-3}\ s^{-1}$ for the 30/M13-mer. Turnover of dCTP acts similarly, where the values are $1.3 \times 10^{-3}\ s^{-1}$ (9/20-mer) versus $6.8 \times 10^{-3}\ s^{-1}$ (30/M13-mer).

Since the kinetics for the 30/M13-mer are similar to those of the 9/20-mer, we conclude that the short DNA duplexes used in our studies of both correct incorporation (Mizrahi et al., 1985; Kuchta et al., 1987) and misincorporation (Kuchta et al., 1988) accurately reflect the mechanism of replication fidelity by pol I in vivo.

KF(polA5). The KF(polA5) mutant carries a point mutation, Gly850Arg (Joyce et al., 1985a), which lies in the DNA binding cleft close to the putative dNTP binding site (Joyce et al., 1985b). Since the introduction of a positively charged side chain might affect dNTP discrimination by the polymerase, we chose to assess the fidelity of KF(polA5) by examining misincorporation of dATP into the 9/20-mer as above.

There was little detectable accumulation of the 9A/20-mer (<2% of the 9/20-mer was converted to 9A/20-mer) whereas under identical conditions KF(exo⁺) accumulated 9A/20-mer to about 50% of the total DNA in the reaction at a rate of $10^{-3}\ s^{-1}$. Additionally, there was no measurable hydrolysis of dATP to dAMP ($<10^{-4}\ s^{-1}$) with KF(polA5) and 9/20-mer, whereas, with KF(exo⁺) under these conditions, the rate of

Table II: Kinetic Parameters of KF(polA5)

	KF(polA5)	KF(exo$^+$)[a]
K_D(13/20-mer) (nM)	25	5
k_{off}(13/20-mer) (s^{-1})	0.7	0.06
k_{pol} (s^{-1})	12	50
K_D(dATP) (μM)	30	5
k_{exo} (s^{-1})[b]	2.0×10^{-3}	1.5×10^{-3}

[a] Data from Kuchta et al. (1987). [b] The exonuclease rate was measured with the 13/20-mer substrate.

dATP hydrolysis was 10^{-3} s^{-1} (Kuchta et al., 1988). However, the exonuclease of the KF(polA5) was functional (Table II), which excluded the possibility that the lack of dATP hydrolysis was due to an inactive exonuclease. Rather, the data indicated that, at least with respect to 9/20-mer + dATP → 9A/20-mer, KF(polA5) was more accurate than wild-type KF(exo$^+$). As a caveat, if the efficiency of misincorporation by KF(polA5) were reduced by the same factor as was the efficiency of correct synthesis (25-fold), the rate of misincorporation by KF(polA5) would only be slightly higher than the limit of detection. A qualitative survey of potential mismatches using the 9/20-mer, 10/20-mer, 13/20-mer, and 14/20-mer revealed that only one of them, misincorporation of A into the 14/20-mer, accumulated to an extent greater than the background level (data not shown).

The potential processivity of the KF(polA5) was examined for dATP incorporation into 13/20-mer by separately quantitating the polymerization rate and the DNA off-rate (Table II). Processivity has been equated to the kinetic partitioning of the polymerase between dissociation from the duplex and nucleotide incorporation (Bryant et al., 1983). The 13/20-mer dissociation rate, which was measured with an isotope trapping experiment of the DNA product (14/20-mer), yielded a value of 0.7 s^{-1} corresponding to an 11-fold increase in this rate relative to that of the KF(exo$^+$). The polymerization rate which was also measured for the incorporation of the correct nucleotide (dATP) with the same DNA yielded a value of 12 s^{-1}, representing a 4-fold decrease in this rate. These data are in accord with direct measurements of the processivity of polymerase by KF(polA5) relative to KF(exo$^+$) (Matson et al., 1978). Therefore, the reduction in processivity by KF(polA5) was achieved through an increase in the 13/20-mer dissociation rate and a decrease in the polymerization rate. Thus, associating increased fidelity with higher processivity does not hold for KF(exo$^+$) and KF(polA5) (Kunkel, 1985).

Kinetic Parameters of KF(exo$^-$). Initial experiments compared pre-steady-state incorporation of dTTP into the 9/20-mer for the KF(exo$^+$) and KF(exo$^-$). Both the burst rate of 50 s^{-1} and K_D(dTTP) equal to 20 μM were identical (data not shown), indicating that the polymerization reaction remains unaffected by mutagenesis of the exonuclease site which is consistent with previous results (Derbyshire et al., 1988). This is also in accord with the X-ray crystallographic structure (Ollis et al., 1985) and with conclusions derived from cross-linked DNA (Cowart et al., 1989) that show the polymerase and exonuclease sites are separated by approximately 30 Å or nine base pairs. Additional control experiments demonstrated that the DNA off-rate value (0.30 s^{-1}) for the 9/20-mer with the KF(exo$^-$) was only slightly faster than with the KF(exo$^+$), 0.17 s^{-1} (Kuchta et al., 1987). Since the KF(exo$^+$) and KF(exo$^-$) exhibited similar kinetic parameters for correct polymerization of dNTPs, we concluded that KF(exo$^-$) would allow us to quantify the contribution of the exonuclease activity to fidelity.

The steady-state misincorporation rates for several nucleotides measured with KF(exo$^-$) are listed in Table III. The

Table III: Misincorporation into DNA[a]

	KF(exo$^+$) (s^{-1})[b]	KF(exo$^-$) (s^{-1})	KF(exo$^-$)-(Mn^{2+}) (s^{-1})[c]
13/20-mer + dCTP[d]	$<6 \times 10^{-6}$	2×10^{-4}	9×10^{-3}
13/20-mer + dTTP	$<7 \times 10^{-6}$	1.6×10^{-4}	5×10^{-3}
13/20-mer + dGTP	$<6 \times 10^{-6}$	1×10^{-4}	1.2×10^{-2}
9/20-mer + dCTP[e]	$<2 \times 10^{-5}$	$<1 \times 10^{-5}$	
9/20-mer + dGTP	$<3 \times 10^{-5}$	2.1×10^{-4}	
9/20-mer + dATP	$<1.1 \times 10^{-3}$	2.2×10^{-3}	

[a] Rates were calculated for pmol of product s^{-1} (pmol of KF)$^{-1}$. [b] Data adapted from Kuchta et al. (1988). [c] In these assays, the $MgCl_2$ was replaced with 0.15 mM $MnCl_2$. [d] The correct nucleotide with 13/20-mer is dATP. [e] The correct nucleotide with 9/20-mer is dTTP.

Table IV: Misincorporation of dATP into the 9/20-mer

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
KF(exo$^+$)[a]	0.0021	8.3	250
KF(exo$^-$)	0.0028	7	400
KF(exo$^-$)($MnCl_2$)	0.21	8	26000

[a] Data from Kuchta et al. (1988).

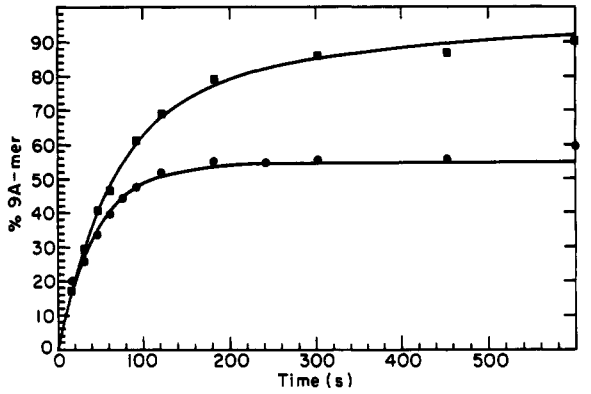


FIGURE 4: Pre-steady-state burst of dATP into the 9/20-mer to form 9A/20-mer. Assays were performed as described previously (Kuchta et al., 1988) with 30 μM dATP, 100 nM 5′-^{32}P-labeled 9/20-mer, and 330 nM either KF(exo$^+$) (●) or KF(exo$^-$) (■). The lines were generated by computer simulation (KINSIM) using the first two stages in Figure 1 and the rate constants in the legend, with k_{exo} set to zero for KF(exo$^-$) and k_{exo} = 1.5×10^{-3} s^{-1} for KF(exo$^+$).

rates increased by a factor of between 2 (9/20-mer + dATP) and 30-fold (13/20-mer + dCTP) consistent with previous work indicating that the exonuclease contributes a factor of ca. 10 to fidelity (Kunkel, 1988). Previously, we had demonstrated a pre-steady-state burst of misincorporation of dATP into 9/20-mer with KF(exo$^+$) (Kuchta et al., 1988). The rate of the burst was dependent on dATP concentration. A similar burst of misincorporation also occurred with the KF(exo$^-$) (Figure 4). As expected, all of the 9/20-mer was converted to the 9A/20-mer when KF(exo$^-$) was used. When the pre-steady-state misincorporation was further examined with 7.5–30 μM dATP, the rate of misincorporation also increased, consistent with a K_M(dATP) value of 7 μM (Table IV).

The steady-state parameters (excess DNA) for misincorporation of dATP into the 9/20-mer were also measured (Table IV). Since the steady-state rate of misincorporation is primarily limited by either k_{exo} or k_{+b}, a comparison of the KF(exo$^+$) and KF(exo$^-$) data provide a direct measure of the fidelity obtained by internal proofreading. The fidelity obtained via internal proofreading is given in eq 4. We have

$$\text{fidelity} = \frac{k_{+b} + k_{exo}}{k_{+b}} \quad (4)$$

omitted the term for the fraction of correct nucleotide removed

Table V: Addition of the Next Correct Nucleotide onto a Mismatch

	KF(exo$^+$)[a,b]		KF(exo$^-$)		KF(exo$^-$)-(Mn^{2+})	
	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)
9C/20-mer + dCTP	0.014	75	0.055	72	0.1	13
9A/20-mer + dCTP	$<2 \times 10^{-5}$		$<5 \times 10^{-5}$		0.008	15
13T/20-mer + dGTP	$<1 \times 10^{-5}$		$<2 \times 10^{-5}$		$<10^{-4}$	

[a] Data from Kuchta et al. (1988). [b] For those cases where k_{cat} and K_M could not be determined, the k_{cat} is the rate measured with 80 μM dNTP [KF(exo$^-$)] or 200 μM dNTP [KF(exo$^+$)].

by the exonuclease activity since the rate of correct polymerization (50 s^{-1}) is much greater than the rate of exonuclease (10^{-3} s^{-1}), so that the latter does not significantly decrease the fraction of correct nucleotides retained. From the net rate constants for 9/20-mer + dATP → 9A/20-mer (Figure 1 legend) and $k_{exo} = 9 \times 10^{-3}$ s^{-1} (Kuchta et al., 1988), the predicted contribution to fidelity by the exonuclease activity is 3.6, while the observed increase is 2.0 (Table III), in reasonable agreement.

Additionally, the contribution of the exonuclease activity of fidelity during the addition of the next correct nucleotide to a mismatch (external proofreading) was also assessed with the KF(exo$^-$) (Table V). Only with the 9C/20-mer was there a measurable increase for the rate of addition of the next correct dNTP—the other rates were too slow to measure. The fidelity obtained through external proofreading is given in eq 5 (Kuchta et al., 1988), where k_{+c} is the rate of polymerization

$$\text{fidelity} = \frac{k_{+c} + k_{exo}}{k_{+c}} \quad (5)$$

onto a mismatched 3′-terminus. The exonuclease rate on the 9C/20-mer (4.5×10^{-3} s^{-1}; Kuchta et al., 1988) and kinetic parameters in Table V for 9C/20-mer + dCTP → 9CC/20-mer predict a 2.2-fold enhancement in fidelity with 5 μM dCTP, while the observed 4-fold enhancement [the ratio of the rates of 9C/20-mer + dCTP → 9CC/20-mer for KF(exo$^-$) and KF(exo$^+$)] is in good agreement.

The 3′→5′ exonuclease activity was therefore shown to contribute to fidelity during both internal and external proofreading. The relatively modest enhancements in fidelity due to proofreading result from the slow rate of the exonuclease. An increased exonuclease rate would have resulted in dramatically increased fidelity. Other key features of the exonuclease activity include the following: (i) the exonuclease rate is too slow, in general, to compete with the rate of correct polymerization; (ii) the specificity parameter (k_{cat}/K_M) for hydrolysis of mismatched and correctly base-paired nucleotides is identical (Kuchta et al., 1988); and (iii) the accuracy of pol I before and after three polymerization cycles remains constant (Kuchta et al., 1988). Consequently, the 3′→5′ exonuclease does not "warm up" as has been suggested (Lecomte & Ninio, 1987).

Mn^{2+}-Induced Mutagenesis. Previous work has demonstrated that Mn^{2+} dramatically decreases the fidelity of pol I (Slater et al., 1972; Weymouth & Leob, 1978; Goodman et al., 1983; El-Diery et al., 1988; Lai & Beattie, 1988). In order to determine the steps that were affected by Mn^{2+}, we used a combination of steady-state and pre-steady-state measurements. Previous studies demonstrated that while Mn^{2+} has little effect on correct polymerization reactions, it does have a small but significant effect on the 3′→5′exonuclease activity (El-Diery et al., 1984, 1988). We therefore used KF(exo$^-$) for these studies to obviate the possibility of exonuclease obscuring effects on the polymerization reaction. As shown in Tables III and V, Mn^{2+} increased the rate of each misincorporation. During misincorporation of dATP into 9/20-mer, Mn^{2+} greatly increased k_{cat} but had little effect on K_M for dATP when compared to Mg^{2+} (Table IV). Replacement of dATP with dATP(αS) resulted in elemental effects on steady-state misincorporation of 32 (k_{cat}) and 39 (k_{cat}/K_M). This contrasted sharply with the elemental effects of 3–7 on steady-state correct dNTP polymerization (Kuchta et al., 1987) and of 5–8 on steady-state misincorporation with Mg^{2+} (Kuchta et al., 1988). These large elemental effects indicate that phosphodiester bond formation is at least partially rate limiting in the steady state during misincorporation with Mn^{2+} as metal cofactor but that the net rate relative to Mg^{2+} (0.21 vs 0.0028 s^{-1}; Table IV) is still 70-fold faster. Rate-limiting phosphodiester bond formation requires that Mn^{2+} not exhibit a pre-steady-state burst of misincorporation into the 9/20-mer as observed with Mg^{2+} (Kuchta et al., 1988). To test this hypothesis, we looked for biphasic kinetics under reaction conditions that contained a 10-fold excess of 9/20-mer over KF(exo$^-$). With either 5 or 10 μM dATP, no burst of 9A/20-mer formation occurred (data not shown) (if all of the KF-9/20-mer were converted to KF-9A/20-mer, a burst of 1 pmol was expected). Furthermore, rates of single turnover and steady-state misincorporation were identical. Finally, Mn^{2+} also increased the rate of polymerization of the next correct nucleotide onto a mismatch when compared to Mg^{2+} (Table V). However, the effect of Mn^{2+} varied remarkably for different DNA substrates (Tables III and V), indicating that the nature of the base pair and perhaps the DNA sequence affects the magnitude of Mn^{2+}-induced mutagenesis.

Conclusions

Studies on the mechanism of KF(exo$^+$), KF(exo$^-$), and KF(polA5) have allowed us to examine more closely the factors which contribute to fidelity of DNA replication by pol I. The lack of nucleotide discrimination originally presented (Kuchta et al., 1988) has now been shown to be present for seven additional mispairs. Additionally, the important features of the mechanism of stable misincorporation extend to longer DNA substrates. The KF(exo$^-$) mutant was used to demonstrate that the exonuclease activity enhances fidelity by as much as a factor of 30. KF(polA5) replicates DNA with decreased processivity due to a decrease in the rate of polymerization and an increase in the rate of release of DNA. As a result, KF(polA5) also appears to have increased fidelity. Finally, Mn^{2+} appears to accelerate all of the steps involved in incorrect synthesis with a concomitant loss of nucleotide discrimination.

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