The Proofreading Pathway of Bacteriophage T4 DNA Polymerase*

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The base analog, 2-aminopurine (2AP), was used as a fluorescent reporter of the biochemical steps in the proofreading pathway catalyzed by bacteriophage T4 DNA polymerase. “Mutator” DNA polymerases that are defective in different steps in the exonucleolytic proofreading pathway were studied so that transient changes in fluorescence intensity could be equated with specific reaction steps. The G255S- and D131N-DNA polymerases can hydrolyze DNA, the final step in the proofreading pathway, but the mutator phenotype indicates a defect in one or more steps that prepare the primer-terminus for the cleavage reaction. The hydrolysis-defective D112A/E114A-DNA polymerase was also examined. Fluorescent enzyme-DNA complexes were preformed in the absence of Mg2+, and then rapid mixing, stopped-flow, stopped-flow techniques were used to determine the fate of the fluorescent complexes upon the addition of Mg2+. Comparisons of fluorescence intensity changes between the wild type and mutant DNA polymerases were used to model the exonucleolytic proofreading pathway. These studies are consistent with a proofreading pathway in which the protein loop structure that contains residue Gly255 functions in strand separation and transfer of the primer strand from the polymerase active center to form a preexonuclease complex. Residue Asp131 acts at a later step in formation of the preexonuclease complex.

Many DNA polymerases achieve a remarkably high level of DNA replication fidelity due, in part, to exonucleolytic proofreading activity (Refs. 1 and 2; reviewed in Refs. 3 and 4). The decision to proofread is determined by the different rates for extension of a correct compared with a mismatched primer-terminus. Nucleotide incorporation is normally rapid from a correct base pair but slow from a mismatched primer-terminus. The slower extension rate in the case of a mismatched primer-terminus provides a window of opportunity to initiate the proofreading pathway.

In order to study DNA polymerase proofreading in real time, we have used the fluorescence of the base analog 2-aminopurine (2AP)† as a reporter. 2AP fluorescence is quenched when 2AP resides in DNA, but fluorescence is restored when the 2AP nucleotide 2AP 2′-deoxyribonucleoside 5′-monophosphate (d2APMP) is released from DNA by DNA polymerase proofreading activity (5–7). These studies revealed a rate-limiting step in the initiation of the proofreading pathway (5–7). This step encompasses separation of the primer strand from the template strand and transfer of the primer strand from the polymerase active center to form a proposed preexonuclease complex (6, 7). A rate of about 4 s−1 is detected for this step for the wild type T4 DNA polymerase with the fluorescence assay, which is consistent with a rate of about 5 s−1 detected with a rapid quench assay (8).

2AP fluorescence is also produced by formation of a specific complex with T4 DNA polymerase in which DNA labeled at the 3′-end with 2AP interacts with amino acid residues in the exonuclease active center (5). Fluorescence anisotropy studies indicate that the fluorescence complex is an intermediate in the proofreading pathway (5). This proposal is supported by studies of “mutator” T4 DNA polymerases that are defective in reaction steps of the exonucleolytic proofreading pathway that prepare the primer-terminus for excision. Two mutant DNA polymerases, the G255S-DNA polymerase (Ser substitution for Gly255) and the D131N-DNA polymerase (Asn substitution for Asp131), have reduced ability to form fluorescent complexes (7).

The rate-limiting step in the proofreading pathway, which is about 4 s−1 for the wild-type T4 DNA polymerase, is reduced 10-fold for the mutant DNA polymerases (6, 7). Residue Gly255 resides in a novel protein loop structure in the exonuclease domain, and this structure is implicated in facilitating movement of the primer-terminus from the polymerase to the exonuclease active center (6). Residue Asp131 resides near the exonuclease active center (7).

The use of the highly sensitive 2AP fluorescence assay and the availability of mutant DNA polymerases with defects in the exonucleolytic proofreading pathway provide the means to equate changes in fluorescence intensity with specific reaction steps. We report here reactions that begin with preformed fluorescent enzyme-DNA complexes that can be trapped in the absence of Mg2+. The addition of Mg2+ to fluorescent complexes formed with the wild type T4 DNA polymerase produced excision product in a rapid, intramolecular reaction; thus, the fluorescent complex is an intermediate or can be converted to an intermediate in the proofreading pathway. Excision product was also produced from complexes formed with the G255S-DNA polymerase but not from complexes formed with the

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† The abbreviations used are: 2AP, 2-aminopurine; d2APMP, 2-aminopurine 2′-deoxyribonucleoside 5′-monophosphate.
D131N-DNA polymerase. These results are consistent with a proofreading scheme in which residues in the Gly\textsuperscript{255}-protein loop function in transfer of DNA from the polymerase active center to form a preexonuclease complex. Residue Asp\textsubscript{131} is also implicated in formation of a preexonuclease complex, but after action of the Gly\textsuperscript{255}-protein loop.

Experiments were also done with the exonuclease-deficient D112A/E114A-DNA polymerase (Ala substitutions for Asp\textsubscript{131} and Glu\textsubscript{114}). Comparisons of fluorescence intensity changes between the wild type and D112A/E114A-DNA polymerases, along with the G255S- and D131N-DNA polymerases, were used to model the exonucleolytic proofreading pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**

Enzymes—The D131N- and G255S-DNA polymerases were identified in genetic selections for mutant DNA polymerases with reduced exonucleolytic proofreading, the mutator phenotype (9, 10). Purification of the wild type and the mutant D131N-, G255S-, and D112A/E114A-DNA polymerases was from the T4 DNA polymerase expression vector (11) has been described in general (12) and specifically (7, 10, 13). The D131N-DNA polymerase has an Asn substitution for Asp\textsubscript{131} (7). The G255S-DNA polymerase has a Ser substitution for Gly\textsuperscript{255} (6, 10). The D112A/E114A-DNA polymerase has Ala substitutions for Asp\textsubscript{131} and for Glu\textsubscript{114} (13).

DNA Substrates—Oligonucleotides labeled with 2AP at the 3'-end were synthesized as described previously using a 2AP-derivatized controlled pore glass solid support (14) or by using a 2AP phosphoramidite (Glen Research, Sterling, VA) and a 3'-phosphate controlled pore glass as support. The synthetic oligonucleotides were purified by polyacrylamide gel electrophoresis using a 14% polyacrylamide, 8 mM urea gel. For oligonucleotides prepared from the 3'-phosphate controlled pore glass support, the 3'-phosphate activity of bacteriophage T4 polynucleotide kinase was used to remove the 3'-terminal phosphate group. The primer was a 17-mer with the sequence 5'-GACGGTACATTACAAGGT- (2AP). Two template DNAs were used: 5'-TGGAGTTAGGCTAGTCT/CAC- CTTAATGACGC. The primer and template DNAs were annealed in buffer containing 25 mM HEPES (pH 7.5) and 50 mM NaCl with a 20% excess of template strand to ensure complete hybridization of the 2AP primer strand (5). The 2AP-T primer-template was prepared by annealing the 2AP primer to the template DNA with T in the variable position. The variable nucleotide is underlined in the sequence of the template strand. The 2AP-C mispair was formed by annealing the 2AP-primer to the DNA template with C in the variable position. DNA primer-templates were as follows.

\[
\begin{align*}
3' &- CGTGCAGTAATTGCA(T/C)GGATCGATGGTTT \\
5' &- GACGGTACATTACAAGGT- (2AP)
\end{align*}
\]

**SEQUENCE 1**

**Methods**

Stopped-flow emission scans and kinetic measurements were performed using an Applied Photophysics SX.17 MV spectrofluorimeter. Excitation was set at 310 nm from a xenon arc lamp. A 335-nm cut-off filter was used. The photomultiplier voltage was set at 700 V, and scattered light was subtracted by applying an offset voltage. All measurements were carried out at 20.0 ± 0.5 °C. The instrument dead time was approximately 1.5 ms. Between six and eight determinations were performed for each reaction, and mean values were calculated. The experimental traces were fit to either single or double exponential equations using the SF 17 MV Kinetic Software Package supplied by Applied Photo-Physics. The agreement between the experimental equations using the SF 17 MV Kinetic Software Package supplied by Applied Photo-Physics. The agreement between the experimental traces and the calculated curves was determined by analysis of the randomness of the distribution in residuals for either single or double exponential equations.

Reactions were initiated in the stopped-flow apparatus by mixing equal volumes of a solution of 1.4 mM T4 DNA polymerase, 400 mM 2AP-DNA substrate, 25 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM dithiothreitol, and 1 mM EDTA with a second solution of 16 mM MgCl\textsubscript{2}, 25 mM HEPES (pH 7.5), and 50 mM NaCl. The final concentrations of reaction components were 700 mM T4 DNA polymerase, 200 mM 2AP-DNA substrate, 25 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, and 8 mM MgCl\textsubscript{2}. In experiments with the heparin trap, heparin (Sigma; catalog no. H5271) was present at 0.2 mg/ml in the syringe with the Mg\textsuperscript{2+} solution; after mixing, the final concentration of heparin was 0.1 mg/ml. No further trapping efficiency was detected when the final concentration of heparin was raised to 0.2 mg/ml. The background level of fluorescence due to buffer components, enzyme, and unbound 2AP-DNA was about 0.15.

**RESULTS**

The Primer-Terminus Resides in the Exonuclease Active Center in Fluorescent Enzyme-DNA Complexes—The experiments presented here begin with preformed fluorescent enzyme-DNA complexes. Several studies indicate that the 3'-2AP-labeled primer-terminus in fluorescent complexes resides in the exonuclease active center. First, mutant T4 DNA polymerases with alanine substitutions for acidic amino acid residues in the exonuclease active center that are required for the hydrolysis reaction are also partially defective in forming fluorescent complexes (5). Second, higher fluorescence intensity is detected with complexes formed with single-stranded or partially single-stranded 3'-labeled 2AP-DNAs, which are more likely to be bound in the exonuclease active center, than for fully duplex DNAs, which are more likely to bind in the polymerase active center (5, 6). Since DNA binding in the polymerase active center does not appear to produce fluorescence, conditions that favor increased partitioning of DNA to the exonuclease active center, which happens with single-stranded DNA, produce more fluorescent complexes. Third, dNTPs and PP\textsubscript{i}, which stabilize primer-terminus binding in the polymerase active center at the expense of DNA binding in the exonuclease active center, also reduce formation of fluorescent complexes. A fourth observation, which is particularly relevant to our studies, is that the absence of Mg\textsuperscript{2+} favors formation of fluorescent complexes, while the presence of Mg\textsuperscript{2+} increases DNA binding in the polymerase active center.

We propose that the environment that is responsible for creating 2AP fluorescence in the T4 DNA polymerase enzyme-DNA complex is linked to how the enzyme prepares the 3'-terminal nucleotide for the hydrolysis reaction. Thus, adding Mg\textsuperscript{2+} to preformed fluorescent complexes may provide insights about further processing of the primer-terminus in preparation for the hydrolysis reaction as well as the cleavage reaction itself. Changes in fluorescence intensity provide the means to follow interactions of the enzyme with the 3'-terminal 2AP nucleotide.

The Addition of Mg\textsuperscript{2+} to Preformed Fluorescent Enzyme-DNA Complexes—In one syringe of the stopped-flow apparatus, fluorescent enzyme-DNA complexes were preformed in the absence of Mg\textsuperscript{2+} under optimal conditions for formation of fluorescent complexes, 3.5-fold excess wild type T4 DNA polymerase to 2AP-DNA (see “Methods”). A solution of Mg\textsuperscript{2+} was contained in the second syringe. When the solutions were mixed, the fluorescence intensity declined rapidly at the apparent rate of about 211 s\textsuperscript{-1} (Fig. 1, Table I). The initial rapid decrease in fluorescence intensity was followed by a biphasic increase in fluorescence intensity at rates of about 3 and 23 s\textsuperscript{-1}. The interesting observation is the detection of a transient, intervening state with reduced fluorescence intensity. Characterization of this intervening state is addressed in the following experiments.

The addition of Mg\textsuperscript{2+} produced a rapid decline in fluorescence intensity to about 0.65, well above the background level of 0.15 (Fig. 1, Fig. 2A). The residual fluorescence could be due to excision and release of the fluorescent product, d2APMP, if excision can take place within the first 20 ms of reaction. In

\[\textsuperscript{2}J. M. Beechem, M. R. Otto, L. B. Bloom, R. Eritja, L. J. Reha-Krantz, and M. F. Goodman, submitted for publication.\]

\[\textsuperscript{2}L. A. Marquez, D. Vijayalakshmi, and L. J. Reha-Krantz, unpublished data.\]
order to test this possibility, the Mg^{2+}-initiated experiments were repeated with an exonuclease-deficient T4 DNA polymerase, the D112A/E114A-DNA polymerase (13). The D112A/E114A-DNA polymerase produces excision product only very slowly, 0.01 s⁻¹ (16); thus, d2APMP production cannot be observed in the first few seconds of reaction. A rapid decline in fluorescence intensity was observed for the D112A/E114A-DNA polymerase when Mg^{2+}− was added to the preformed fluorescent enzyme-DNA complexes, but the apparent rate was slower than observed with the wild type enzyme, about 70 s⁻¹ (Fig. 2B; Table I). A greater decrease in intensity of the fluorescent signal was also observed for the exonuclease-deficient enzyme than for the wild type enzyme (Fig. 2, compare A and B). The end point reached in the first 100 ms of reaction for the exonuclease-deficient enzyme was about 0.3. This value, lower than observed for the wild-type enzyme, indicates that the higher level of fluorescence intensity observed for the wild-type enzyme after the initial rapid decline may be the result of excision and production of fluorescent d2APMP. The 0.3 value reached by the exonuclease-deficient D112A/E114A-DNA polymerase, on the other hand, may be due to reformation of fluorescent complexes, but at a lower level in the presence of Mg^{2+}− than in the absence of Mg^{2+}−.

The 2AP-DNA substrate used for experiments in Fig. 2, A and B, has 2AP in the S′-terminal position paired opposite template T (2AP-T DNA). The Mg^{2+}-initiated experiments were repeated with the 2AP-C DNA substrate, in which 2AP is paired opposite template C (Fig. 2, C and D). The end points following the rapid initial decline in fluorescence intensity were higher with the 2AP-C DNA substrate than for the 2AP-T substrate, particularly for the D112A/E114A-DNA polymerase (compare the fluorescence decay end point of about 0.3 for the 2AP-T substrate to 0.65 for the 2AP-C substrate (Fig. 2, B and D)). A higher level of fluorescent complex formation is predicted for the mispaired 2AP-C DNA than the 2AP-T DNA, since a mispaired primer-terminus is less likely to be bound in the polymerase active center. For the wild type enzyme, excision and fluorescent complex formation are both likely possibilities to account for the level of fluorescence reached after the initial decline.

Formation of Fluorescent Enzyme-DNA Complexes in the Presence of Mg^{2+}−—In stopped-flow experiments in which a solution of 2AP-T DNA was mixed with a solution of exonuclease-deficient D112A/E114A-DNA polymerase and Mg^{2+}− (association experiments), an increase in fluorescence intensity was observed from the background level of 0.15 to about 0.3 at the apparent rate of 80 s⁻¹ (Fig. 3). This rate corresponds to a bimolecular association rate of about 1 × 10⁻⁸ M⁻¹ s⁻¹ as observed previously (5, 8). The increase in fluorescence intensity is due to formation of the fluorescent complex, since the mutant enzyme is severely defective in the hydrolysis reaction. The steady-state level of fluorescence intensity of 0.3 corresponds to the end point reached in the fluorescent decay curve in the Mg^{2+}-initiated experiments with 2AP-T DNA (Fig. 2B).

Fluorescent complex formation was also measured for the exonuclease-deficient D112A/E114A-DNA polymerase with the 2AP-C DNA substrate. An approximate 2-fold higher steady-state level of fluorescent complex was formed with the 2AP-C DNA (0.65) than with the 2AP-T DNA (Fig. 3), but the apparent association rate, 92 s⁻¹, was similar to the rate observed with the 2AP-T DNA. The higher steady-state level of fluorescent complex formed with the 2AP-C DNA than with the 2AP-T DNA is consistent with previous results in which a larger population of fluorescent enzyme-DNA complexes was formed with a single-stranded 2AP-DNA compared with a double-stranded 2AP-DNA (5) and with duplex DNAs with preformed terminal strand separations.4 As observed with the 2AP-T DNA substrate, the steady-state level of fluorescence detected for complex formation with the 2AP-C DNA corresponds to the fluorescence decay end point in the Mg^{2+}-initiated experiments (Fig. 2D).

Thus, the rapid decline in fluorescence intensity upon the addition of Mg^{2+}− with the exonuclease-deficient DNA polymerase appears to be followed by rapid reformation of fluorescent complexes, but the level of fluorescence is below that obtained in the absence of Mg^{2+}−. The steady-state level of fluorescence intensity reached with the 2AP-T DNA in the absence of Mg^{2+}− was about 0.9, while the end point reached in the presence of Mg^{2+}− was about 0.3 (Fig. 3). This difference is the basis for the decline in fluorescence intensity observed when Mg^{2+}− is added to complexes formed in the absence of Mg^{2+}− (Fig. 2B). The decreased level of fluorescence intensity is due to increased formation of nonfluorescent enzyme-DNA complexes when Mg^{2+}− is added, such as complexes in which the 3′-2AP-labeled primer-terminus is bound in the polymerase active center.2

Experiments with a Heparin Trap—The above experiments suggest that the addition of Mg^{2+}− to preformed fluorescent enzyme-DNA complexes results in a rapid decrease in fluorescence intensity that is coupled to an increase in fluorescence intensity that may be due to either excision and release of the fluorescent d2APMP, in the case of the wild-type enzyme, or to reformation of fluorescent complexes at a reduced level, as seen for the exonuclease-deficient DNA polymerase. A heparin trap can be used to determine if the wild-type enzyme carries out the excision reaction. Heparin traps T4 DNA polymerase as the enzyme dissociates from the 2AP-DNA substrate and thus prevents reformation of enzyme-DNA complexes (17, 18). Thus,

4 L. A. Marquez and L. J. Reha-Krantz, unpublished data.
fluorescence intensity will decline from enzyme-DNA complexes as DNA polymerase molecules dissociate from the 2AP-DNA and are trapped by heparin. The fluorescence of d2APMP, however, is not sensitive to the heparin trap.

When a solution of fluorescent enzyme-DNA complexes formed with the wild type T4 DNA polymerase was mixed with a solution of Mg$$^{2+}$$ and heparin, a decline in fluorescence intensity was detected that was similar to the decline observed without the heparin trap for the first 20 ms of reaction. Compare the Mg$$^{2+}$$-initiated reaction with 2AP-T DNA in Fig. 2A without heparin to the reaction in Fig. 4A with heparin. Also compare the reactions with the 2AP-C DNA substrate, Fig. 2C (no heparin) with Fig. 4C (with heparin). The level of fluorescence produced after the initial decline was insensitive to heparin, which indicates that this fluorescence is due to production of fluorescent d2APMP rather than to the fluorescent complexes. The increase in fluorescence intensity observed with the wild type enzyme at longer reaction times (Fig. 1) was not seen in the presence of the heparin trap. Thus, the changes in fluorescence intensity in Fig. 1 can be explained in two parts. Initially, a rapid decline in fluorescence intensity is produced by Mg$$^{2+}$$ binding. About 70% of the initial complexes produce a burst of excision product via an intramolecular pathway while the remaining 30% dissociate. In the second part, which is observed in the presence of heparin, a decline in fluorescence intensity is insensitive to heparin trap. Thus, the changes in fluorescence decay end points reached with the heparin trap for both DNA substrates were lower than without the trap and reached an intensity of about 0.2, which is approximately the level of background fluorescence. Moreover, fluorescence decay curves in the presence of heparin, unlike the rates detected in the absence of heparin, were biphasic. The best curve fit for the experimental traces for the 2AP-T DNA substrate (Fig. 4B) was achieved by using a double exponential equation with decay rates of about 100 s$$^{-1}$$ and 12 s$$^{-1}$$ (Table I). Apparent rates of 85 and 15 s$$^{-1}$$ were observed for the 2AP-C DNA substrate (Fig. 4D). The faster rates were detected in the absence of the heparin trap, but the slower rates of 12–15 s$$^{-1}$$ probably measure an additional reaction step that could be observed only in the presence of the trap. We propose that the slower rates measure the dissociation of enzyme from the 2AP-DNA substrate. The proposed dissociation rates are similar to the dissociation rates measured for the wild type T4 DNA polymerase by Capson et al. of 6–8 s$$^{-1}$$ (8) determined by a rapid quench method.

**Mutant T4 DNA Polymerases Defective in Proofreading**—Alanine substitutions for residues Asp$^{112}$ and Glu$^{114}$ are proposed to severely inhibit the ability of the enzyme to bind Mg$$^{2+}$$ ions in the exonuclease active center that are required for the hydrolysis reaction (reviewed in general in Ref. 19; see Refs. 20 and 21 for descriptions of the T4 DNA polymerase exonuclease active center). Thus, when Mg$$^{2+}$$ is added to preformed fluorescent complexes with the D112A/E114A-DNA polymerase, the decline in fluorescence intensity cannot be due to binding of Mg$$^{2+}$$ in the exonuclease active center. Instead, the decline in fluorescence intensity at the rate of 70–100 s$$^{-1}$$ (Table I) for the D112A/E114A-DNA polymerase must be due to Mg$$^{2+}$$ binding via the heparin insensitive pathway cannot be determined directly by inspection of the curves that show the rapid decline in fluorescent intensity (Fig. 4, A and C), because these curves represent the sum of multiple reactions, reactions that reduce and increase the fluorescent signal. A breakdown of the individual reaction steps in the first 50 ms of reaction is described under “Discussion” and in the Appendix.

For the exonuclease-deficient D112A/E114A-DNA polymerase, the addition of the heparin trap changed both the apparent decay rates and the end points of fluorescence decay in Mg$$^{2+}$$-initiated experiments. Compare reactions with the 2AP-T DNA substrate in Fig. 2B (no heparin) and Fig. 4B (with heparin) and reactions with 2AP-C DNA in Fig. 2D (no heparin) to Fig. 4D (with heparin). The fluorescence decay end points reached with the heparin trap for both DNA substrates were lower than without the trap and reached an intensity of about 0.2, which is approximately the level of background fluorescence. Moreover, fluorescence decay curves in the presence of heparin, unlike the rates detected in the absence of heparin, were biphasic. The best curve fit for the experimental traces for the 2AP-T DNA substrate (Fig. 4B) was achieved by using a double exponential equation with decay rates of about 100 s$$^{-1}$$ and 12 s$$^{-1}$$ (Table I). Apparent rates of 85 and 15 s$$^{-1}$$ were observed for the 2AP-C DNA substrate (Fig. 4D). The faster rates were detected in the absence of the heparin trap, but the slower rates of 12–15 s$$^{-1}$$ probably measure an additional reaction step that could be observed only in the presence of the trap. We propose that the slower rates measure the dissociation of enzyme from the 2AP-DNA substrate. The proposed dissociation rates are similar to the dissociation rates measured for the wild type T4 DNA polymerase by Capson et al. of 6–8 s$$^{-1}$$ (8) determined by a rapid quench method.
were initiated by the addition of a solution of Mg²⁺ and heparin to a second solution containing fluorescent enzyme-DNA complexes preformed in the absence of Mg²⁺. Time courses for reactions with the wild type (WT) T4 DNA polymerase are presented in A and C, and time courses for the exonuclease-deficient D112A/E114A-DNA polymerase are presented in B and D. Fluorescent enzyme-DNA complexes were formed with the 2AP-T DNA substrate (A and B) and with the 2AP-C DNA substrate (C and D).

in the polymerase active center and at other potential sites on the enzyme and DNA substrate, which shifts the equilibrium between fluorescent and nonfluorescent complexes to increased formation of nonfluorescent species. The reduced ability to bind Mg²⁺ in the exonuclease active center by the D112A/E114A-DNA polymerase may account for the slower rate of fluorescence decline detected with the mutant enzyme (70–100 s⁻¹) compared with the wild type enzyme (211–249 s⁻¹) (Table I). While the wild type enzyme can carry out the excision reaction upon binding Mg²⁺ in the exonuclease active center, the exonuclease-deficient D112A/E114A-DNA polymerase is restricted to moving the primer-terminus from the exonuclease active center to produce nonfluorescent species, such as to the polymerase center or to dissociation of the complex. The faster rate for fluorescence decline detected for the wild type enzyme indicates that Mg²⁺ binding in the exonuclease active center produces additional changes in conformation that reduce fluorescence intensity.

The G255S-DNA polymerase, is also defective in proofreading, but this mutant enzyme can bind Mg²⁺ in the exonuclease active center and can catalyze the hydrolysis reaction, since nearly wild type levels of exonuclease activity are observed with single-stranded DNA substrates and with duplex DNAs with preformed terminal strand separations (6). Reduced activity, however, is detected with fully duplex DNA substrates such as the 2AP-T DNA substrate. We proposed previously (6) that an initial rate-determining step in the proofreading pathway is formation of a partially strand-separated, preexonuclease complex. Formation of the proposed preexonuclease complex is 10-fold slower for the G255S-DNA polymerase than for the wild type enzyme (6). In order to determine if subsequent steps in the proofreading pathway are also affected by the G255S-DNA polymerase, Mg²⁺ was added to fluorescent complexes preformed in the absence of Mg²⁺. A rapid decline in fluorescence intensity at the apparent rate of 291 s⁻¹ was observed (Table I). Excision product was also detected during the fluorescence decay process as observed for the wild type enzyme. These results indicate that the G255S-DNA polymerase is not defective in reaction steps that follow formation of the fluorescent complex.

The D131N-DNA polymerase is also defective in proofreading (9). As observed for the G255S-DNA polymerase, formation of the proposed preexonuclease complex is about 10-fold slower (7, 16). When Mg²⁺ was added to complexes formed with the D131N-DNA polymerase in the absence of Mg²⁺, the apparent rate of fluorescence decay, 71 s⁻¹, was similar to the rate detected for the D112A/E114A-DNA polymerase (Table I), and little excision product was detected. Thus, the fluorescent complexes formed by the D131N-DNA polymerase do not appear to be competent to carry out the excision reaction. Since normal excision activity was observed with fluorescent complexes formed with the G255S-DNA polymerase, the D131N substitution appears to affect a step in the proofreading pathway that follows the step affected by the G255S substitution. This proposal is consistent with the location of Asp¹³¹ near the exonuclease active center (7, 20).

DISCUSSION

Previous experiments with ³²P-labeled DNA substrates by Capson et al. (8) demonstrated that the addition of Mg²⁺ to T4 DNA polymerase-DNA complexes preformed in the absence of Mg²⁺ produced a burst of excision product at the apparent rate of 100 s⁻¹. The burst of excision activity was from a population of enzyme-DNA complexes activated by hydrolytic cleavage. Our experiments show that preformed T4 DNA polymerase-DNA complexes are fluorescent when the primer DNA is labeled at the 3’ terminus with 2AP. As observed with the ³²P-labeled DNA substrates, a burst of excision product was produced when Mg²⁺ was added to the preformed fluorescent complexes along with a rapid decline in fluorescence intensity (Figs. 1, 2, and 4; Table I). The decline in fluorescence intensity was more rapid for the wild type and G255S-DNA polymerases than for the D112A/E114A- and D131N-DNA polymerases.

The addition of Mg²⁺ initiates several reactions: Mg²⁺ binding in the exonuclease and polymerase active centers, a redistribution of the primer-terminus between the polymerase and exonuclease active centers, formation of complexes activated for hydrolysis, and finally excision of the terminal 2AP nucleotide. These reaction steps are modeled in Schemes 1–3.
less fluorescent state II were analyzed. We also begin our modeling with the D112A/E114A-DNA polymerase, which does not produce excision product. The addition of the heparin trap, which prevents enzyme reassociation with DNA, further simplifies modeling of the reaction. The biochemical steps that describe the addition of Mg$^{2+}$ to the fluorescent complex preformed with the D112A/E114A-DNA polymerase and the 2AP-T DNA substrate are given in Scheme 1. The rate of decline in fluorescence intensity from state I to state II for the D112A/E114A-DNA is $k_{fd(exo)}$.

The highest fluorescence signal with the exonuclease-deficient enzyme was detected for the fluorescent complexes formed in the absence of Mg$^{2+}$ (state I), and this level was normalized to 1.0. The absence of Mg$^{2+}$ favors binding of the 2AP primer-terminus in the exonuclease active center, and excess enzyme ensures that all of the DNA is bound by enzyme. Upon the addition of Mg$^{2+}$, there was a decrease in fluorescence intensity to about 25% of the initial level (state II). This value was calculated from the steady-state levels of fluorescent complex formed in the absence and presence of Mg$^{2+}$ for the D112A/E114A-DNA polymerase and the 2AP-T DNA substrate, less the background level of fluorescence (Fig. 3). The reduced level of fluorescence in the presence of Mg$^{2+}$ (state II) indicates a redistribution of the 2AP-DNA between fluorescent states and states with reduced or no fluorescence. For example, the addition of Mg$^{2+}$ increases binding of the primer-terminus in the polymerase-DNA complex and this polymerase-DNA complex is not fluorescent. A further decline in fluorescence intensity was detected with the heparin trap due to dissociation of the enzyme from the fluorescent complex at the apparent rate of about 12 s$^{-1}$ (Fig. 4B; Table I).

Since experimental values have been obtained for the indicated parameters, it is possible to find the rate of fluorescence decline, $k_{fd(exo)}$, from the fluorescent state I to the less fluorescent state II. Equations describing the kinetic scheme are presented in the Appendix. Changes in fluorescence intensity for each of the reactions are illustrated by the curves in Fig. 5A. The rate of fluorescence decline is described by curve $d$, and curve $f$ describes the changes in fluorescence intensity for state II, both formation and decline due to dissociation. A close fit of the modeled summation curve, curves $d$ + $f$, with the observed experimental data was achieved when $k_{fd(exo)}$ was equal to 90 s$^{-1}$, a value close to the observed rate of 100 s$^{-1}$ detected with the heparin trap (Fig. 4B; Table I).

Changes in fluorescent intensity in the absence of heparin (Fig. 2B) could also be modeled using the same scheme except that enzyme reassociation at the rate of 80 s$^{-1}$ was included (analysis not shown).

**Modeling the Proofreading Pathway for Wild Type T4 DNA Polymerase**—The same approach used for the exonuclease-deficient D112A/E114A-DNA polymerase was used for the wild type DNA polymerase except that the hydrolysis reaction was included. The proposed proofreading pathway, starting with the highly fluorescent state I, is given in Scheme 2. The addition of the heparin trap limits the reactions detected to intramolecular events. The heparin trap did not appear to significantly affect the initial stages of the reaction, since the rates of fluorescence decline and the end points reached were similar in the presence or absence of heparin (compare Fig. 2A with Fig. 4A; Table I). The rate of fluorescence decline for the wild type enzyme is given by $k_{fd(wt)}$.

The faster apparent rate of fluorescent decline for the wild type T4 DNA polymerase compared with the exonuclease-deficient D112A/E114A-DNA polymerase (Table I) indicates that Mg$^{2+}$ binding in the exonuclease active center of the wild type enzyme provides an additional mechanism of fluorescence quenching not possible for the mutant. Thus, the fluorescence
Details of the reaction scheme are presented in the Appendix.

experiments. When a solution containing enzyme and Mg\textsuperscript{2+} forms the preexonuclease complex. We do not think that another possibility is that this rate measures formation of a fluorescent complex, which the DNA is bound directly from preformed fluorescent complexes. Formation of excision product accounts for the higher fluorescence decay end point observed for the wild type enzyme compared with the exonuclease-deficient D112A/E114A-DNA polymerase. The hydrolysis rate for wild type T4 DNA polymerase has been measured by a rapid quench method and was found to be about 100 s\textsuperscript{-1} (8) and about 80 s\textsuperscript{-1} for 2AP-labeled single-stranded DNA (5). Dissociation, however, was detected for the exonuclease-deficient D112A/E114A-DNA polymerase at about 12 s\textsuperscript{-1} (Scheme 1). Dissociation was also detected for the wild type enzyme, since only about 70% of the preformed fluorescent complexes were able to produce excision product in the presence of the heparin trap. If dissociation by the wild type enzyme is also at about 12 s\textsuperscript{-1}, then an intervening reaction step before the hydrolysis reaction is indicated. Modeling of the reaction steps for the wild type enzyme in the presence of the heparin trap suggests that a second complex is formed at the calculated rate, \(k_c\), of about 20 s\textsuperscript{-1} (see Appendix). Thus, we propose that \(k_c\) is the rate of formation of an additional fluorescent state, state III. Excision then proceeds from state III.

Each of the reaction steps in Scheme 2, in the presence of the heparin trap, produces the changes in fluorescence intensity described by the curves in Fig. 5B. Curves \(d, f, k_c\), and \(p\) represent changes in fluorescence intensity for state II, state I, state III, and d2APMP, respectively. The curve describing the fluorescence arising from the sum of these reactions, \(d + f + k_c + p\), is a good fit to the experimental data with \(k_{d(wt)} = 200 \text{ s}^{-1}\). Details of the reaction scheme are presented in the Appendix.

Support for the \(k_c\) rate in Scheme 2 is provided by other experiments. When a solution containing enzyme and Mg\textsuperscript{2+} is mixed with a solution of 2AP-T DNA in the stopped-flow apparatus, apparent excision rates of 3 and 23 s\textsuperscript{-1} were detected in experiments reported here (Fig. 1), and rates of 4 and 17 s\textsuperscript{-1} were detected previously (6). The slower of the two rates is the \(k_c\) rate in Scheme 2, but other schemes may also be proposed.

Conclusions—We show here that excision product can be produced directly from preformed fluorescent complexes by the wild type T4 DNA polymerase. This observation is consistent with formation of at least one preexonuclease complex intermediate in the proofreading pathway. Analysis of mutant DNA polymerases defective in proofreading, but not in hydrolysis of the phosphodiester bond, can provide more information about the proofreading pathway. In the presence of Mg\textsuperscript{2+}, a rate-limiting step has been measured at rates between 3 and 5 s\textsuperscript{-1} for the wild type T4 DNA polymerase (Refs. 6–8; Fig. 1) and at a 10-fold slower rate for the G255S- and D131N-DNA polymerases (6, 7). The 10-fold slower rate detected for the mutant DNA polymerases is proposed to form a higher kinetic barrier to initiating the proofreading pathway and, thus, explains the mutator phenotype observed for these mutants in vivo. Although the G255S-DNA polymerase is defective in hydrolyzing double-stranded DNA substrates, this mutant is not defective in hydrolyzing duplex DNA bound in preformed fluorescent complexes (Table I). Thus, the G255S substitution appears to affect formation of the preexonuclease complex, but once the preexonuclease complex is formed the complexes are competent to carry out subsequent step(s) in the proofreading pathway. Excision product, however, was not produced from fluorescent complexes formed by the D131N-DNA polymerase; hence, this mutant affects an aspect of fluorescent complex formation with duplex DNAs distinct from the defect caused by the G255S substitution. Residue Gly\textsuperscript{255} resides in a novel protein loop structure in the exonuclease domain (6), while Asp\textsuperscript{131} resides near the exonuclease active center (7). Thus, the Gly\textsuperscript{255}-protein loop may affect strand transfer and separation required to form the preexonuclease complex, while residue Asp\textsuperscript{131} functions in a later stage, perhaps in positioning the primer-terminus in the exonuclease active center. A minimal pathway for proofreading in vivo is illustrated in Scheme 3.

A prediction from Scheme 3 is that if extension is slower than about 3–5 s\textsuperscript{-1}, then the primer-terminus will be separated from the template strand and transferred from the polymerase active center to form a preexonuclease complex. The 3–5 s\textsuperscript{-1} rate was measured for the wild type T4 DNA polymerase with the G + C-rich, 2AP-T DNA substrate, but the rate is increased to 11 s\textsuperscript{-1} for the A + T-rich, 2AP-T DNA (6) and to 12 s\textsuperscript{-1} for the G + C-rich, mispaired 2AP-C DNA substrate.\textsuperscript{5} Thus, instability at the primer-terminus produced by A + T richness or a mispair increases the apparent rate of formation of the preexonuclease complex. An accompanying decrease in the rate of extension would divert the DNA polymerase from primer elongation to the proofreading pathway.

Transfer of the primer-terminus from the polymerase active center to form the preexonuclease complex requires the action of the Gly\textsuperscript{255}-protein loop and then participation of residue Asp\textsuperscript{131}. Formation of a second complex may then follow, and finally there is hydrolysis and release of the terminal nucleotide. The availability of the G255S- and D131N-DNA poly-

\[ \text{Enzyme} + \text{DNA} \rightarrow \text{extension} \]

\[ \text{G255} \downarrow \quad k_c \sim 3 - 5 \text{ s}^{-1} \]

\[ \text{D131} \downarrow \]

\[ \text{Enzyme} + \text{exonuclease} \rightarrow \text{DNA} \quad k_c \sim 17 - 23 \text{ s}^{-1} \]

\[ \text{Enzyme} + \text{exonuclease} \rightarrow \text{DNA} \quad k_c \sim 100 \text{ s}^{-1} \]

\[ \text{Enzyme} + \text{DNA}_1 + \text{2APMP} \]

**Scheme 3. DNA polymerase proofreading pathway.**

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\[ ^{5} \text{L. A. Marquez, R. P. Baker, and L. J. Reha-Krantz, unpublished observations.} \]
merases and other mutant T4 DNA polymerases (9, 10, 15, 18, 22) combined with highly sensitive 2AP-fluorescence assays provide the means to test this scheme and to dissect the proofreading pathway in even greater detail.

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APPENDIX

Processes occurring in the system described in Scheme 1 for the exonuclease-deficient D112A/E114A T4 DNA polymerase in the presence of heparin were modeled by the differential equations,

\[ \frac{dy_1(t)}{dt} = -k_{\text{off}}y_1(t) \]  
\[ \frac{dy_2(t)}{dt} = k_{\text{off}}y_1(t) - k_{\text{fd}}y_2(t) \]  
\[ \frac{dy_3(t)}{dt} = k_{\text{off}}y_2(t) \]

where \( y_1(t), y_2(t), \) and \( y_3(t) \) are probabilities of the system to be in the state I (enzyme-DNA no Mg\(^{2+}\)) state II (enzyme-DNA + Mg\(^{2+}\)), and in the dissociated state (enzyme + DNA), respectively. \( k_{\text{off}} \) is the experimentally determined dissociation constant of 12 s\(^{-1}\). Since heparin was present in the system, no reassociation was possible, and it was not a factor in modeling the reactions. The rate constant \( k_{\text{fd}} \) is the decay rate of the highly fluorescent state I to the less fluorescent state II for the exonuclease-deficient D112A/E114A DNA polymerase. The equations were solved analytically for \( y_1(t), y_2(t), \) and \( y_3(t) \). The fluorescence intensity of the system at any given time moment \( t \) was considered to be proportional to \( y_1(t) + 0.25y_2(t) \), which reflects the fact that the fluorescence level of D112A/E114A enzyme-DNA complex in the presence of Mg\(^{2+}\) is about 25% of the fluorescence level of the same complex in the absence of Mg\(^{2+}\) (see Fig. 3). Curve \( d \) corresponds to \( y_1(t) \), and curve \( f \) corresponds to 0.25\( y_2(t) \) (Fig. 5A). The 2AP-DNA produced by dissociation is not shown, because the quantum yield for the duplex DNA is much less than the quantum yield of the complex and the excision product, d2APMP. The sum of curves \( d \) and \( f \) matches the decline in the fluorescent intensity detected experimentally when \( k_{\text{fd}} \) is equal to 90 s\(^{-1}\). The data in Fig. 4B were normalized in Fig. 5A by equating the initial high level of fluorescence intensity detected for the enzyme-DNA complex formed in the absence of Mg\(^{2+}\) to 1.0. Processes occurring in the system described by Scheme 2 for the wild type T4 DNA polymerase in the presence of heparin (no reassociation) were modeled by the differential equations,

\[ \frac{dy_1(t)}{dt} = -k_{\text{off}}y_1(t) \]  
\[ \frac{dy_2(t)}{dt} = k_{\text{off}}y_1(t) - (k_{\text{off}} + k_{\text{fd}})y_2(t) \]  
\[ \frac{dy_3(t)}{dt} = k_{\text{off}}y_2(t) \]

where \( y_1(t), y_2(t), y_3(t), \) and \( y_4(t) \) are probabilities of the system to be in state I (enzyme-DNA no Mg\(^{2+}\)), state II (enzyme-DNA + Mg\(^{2+}\)), the dissociated state (enzyme + DNA), state III, and the enzyme-DNA\(_{p}\) \( + + \) + 22PMP\(^{2-}\) state, respectively. The rate constant \( k_{\text{off}} \) is the rate of decay of state I to state II for the wild type T4 DNA polymerase. \( k_{\text{fd}} \) is the rate of product formation, and \( k_c \) is the calculated rate of formation of state III. This set of equations was solved analytically for \( y_1(t), y_2(t), y_3(t), \) and \( y_4(t) \). The quantum yield of free d2APMP is 1.1 times higher than the quantum yield of the enzyme-DNA complex in the absence of Mg\(^{2+}\) as calculated from Fig. 1. The fluorescence levels of state II (enzyme-DNA + Mg\(^{2+}\)) and state III are unknown, but were calculated to be 70% of the state I (enzyme-DNA no Mg\(^{2+}\)) fluorescence level by fitting the theoretical curve to the experimental curve. Thus, the fluorescence intensity of the system at any given time moment \( t \) was considered to be proportional to \( y_1(t) + 0.7y_2(t) + 0.7y_3(t) + 1.1y_4(t) \). Curve \( d \) corresponds to \( y_1(t) \), curve \( f \) to 0.7\( y_2(t) \), curve \( c \) to 0.7\( y_3(t) \), and curve \( p \) to 1.1\( y_4(t) \) (Fig. 5B).

The sum of curves \( d, f, c, \) and \( p \) matches the experimental data with \( k_{\text{off}} \) equal to 200 s\(^{-1}\). The data from Fig. 4A were normalized in Fig. 5B by equating the initial high level of fluorescence intensity observed for the enzyme-DNA complex formed in the absence of Mg\(^{2+}\) to 1.0.

REFERENCES

1. Muzyrcka, N., Poland, R. L., and Bessman, M. J. (1972) J. Biol. Chem. 247, 7116–7122.
2. Brutlag, D., and Kornberg, A. (1972) J. Biol. Chem. 247, 241–248.
3. Johnson, K. A. (1993) J. Biol. Chem. 268, 247–252.
4. Bloom, L. B., Otto, M. R., Eritja, R., Reha-Krantz, L. J., Goodman, M. F., and Beechem, J. M. (1994) Biochemistry 33, 7576–7586.
5. Bloom, L. B., Otto, M. R., Eritja, R., Reha-Krantz, L. J., Goodman, M. F., and Beechem, J. M. (1994) Biochemistry 33, 7576–7586.
6. Marquez, L. A., and Reha-Krantz, L. J. (1996) J. Biol. Chem. 271, 28093–28091.
7. Baker, R. P., and Reha-Krantz, L. J. (1998) Proc. Natl. Acad. Sci. U. S. A., 95, 3507–3512.
8. Capson, T. L., Peliska, J. A., Kaoberd, B. F., Frey, M. W., Lively, C., Dahlberg, M., and Benkovic, S. J. (1992) Biochemistry 31, 10984–10994.
9. Reha-Krantz, L. J. (1988) J. Mol. Biol. 202, 711–724.
10. Stocki, S. A., Nonay, R. L., and Reha-Krantz, L. J. (1995) J. Mol. Biol. 254, 15–20.
11. Lin, T. C., Rush, J. R., Spiezer, E. K., and Konigsberg, W. H. (1985) Proc. Natl. Acad. Sci. U. S. A., 84, 7000–7004.
12. Reha-Krantz, L. J., Nonay, R. L., and Stocki, S. (1993) Virology 67, 60–66.
13. Reha-Krantz, L. J., and Nonay, R. L. (1993) J. Biol. Chem. 268, 27169–27160.
14. Eritja, R., Kaplan, B. E., Mhaskar, D., Sowers, L. C., Petruska, J., and Goodman, M. F. (1986) Nucleic Acids Res. 14, 5869–5884.
15. Reha-Krantz, L. J. (1985) Methods Enzymol. 262, 223–331.
16. Baker, R. (1996) Biochemistry 35, 1087–1099.
17. Reha-Krantz, L. J. (1994) in Molecular Biology of Bacteriophage T4 (Karam, J. ed), pp. 307–312, American Society for Microbiology, Washington D. C.