Using 2-Aminopurine Fluorescence and Mutational Analysis to Demonstrate an Active Role of Bacteriophage T4 DNA Polymerase in Strand Separation Required for 3′ → 5′-Exonuclease Activity*

Leah A. Marquez and Linda J. Reha-Krantz‡

From the Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

The fluorescence of 2-aminopurine deoxynucleotide positioned in a 3′-terminal mismatch was used to evaluate the post-steady state kinetics of the 3′ → 5′ exonuclease activity of bacteriophage T4 DNA polymerase on defined DNA substrates. DNA substrates with one, two, or three preformed terminal mispairs simulated increasing degrees of strand separation at a primer terminus. The effects of base pair stability and local DNA sequence on excision rates were investigated by using DNA substrates that were either relatively G + C- or A + T-rich. The importance of strand separation as a prerequisite to the hydrolysis of a terminal nucleotide was demonstrated by using a unique mutant DNA polymerase that could degrade single-stranded but not double-stranded DNA, unless two or more 3′-terminal nucleotides were unpaired. Our results led us to conclude that the reduced exonuclease activity of this mutant DNA polymerase of duplex DNA substrates is due to a defect in melting the primer terminus in preparation for the excision reaction. The mutated amino acid (serine substitution for glycine at codon 255) resides in a critical loop structure determined from a crystallographic study of an amino-terminal fragment of T4 DNA polymerase. These results suggest an active role for amino acid residues in the exonuclease domain of the T4 DNA polymerase in the strand separation step.

Exonucleolytic proofreading by DNA polymerases is an important component of high fidelity DNA replication. Nucleotide insertion errors occur at a frequency of 10^-3 to 10^-5, but the DNA polymerase-associated 3′ → 5′-exonuclease activity further reduces replication errors by 100-fold or more (reviewed in Ref. 1). Different steps of the proofreading reaction have been revealed by mutational analysis of the bacteriophage T4 DNA polymerase. Mutant T4 DNA polymerases deficient in 3′ → 5′-exonuclease activity exhibit a strong mutator phenotype that can be used to select for mutant DNA polymerases (2–4). The loss of the 3′ → 5′-exonuclease activity by mutation can be achieved by preventing distinct steps in the proofreading process. The hydrolysis reaction may be prevented if residues required for catalysis are substituted with other amino acids. For example, the substitution of Ala residues for Asp residues that bind essential Mg2+ ions in the exonuclease active center reduces the exonuclease activity to barely detectable levels (5–7). Another reaction step in proofreading that may be affected by mutation is the translocation of the primer end of the DNA between the spatially distinct polymerase and exonuclease active centers. Identification of several “active-site switching mutants” has been described recently (8, 9). DNA polymerase proofreading is a dynamic process; if “switching” to the exonuclease active site is reduced by mutation, there is less opportunity for proofreading and a mutator phenotype is produced (9).

Another proofreading reaction step that may be probed by mutational analysis of T4 DNA polymerase is strand separation. The 3′ → 5′-exonuclease activity of T4 DNA polymerase (10) and other proofreading DNA polymerases is a single-stranded DNA exonuclease activity. Structural studies of the Klenow fragment (KF) of Escherichia coli DNA polymerase I show that single-stranded DNA is bound in the exonuclease active center (11), and time-resolved fluorescence experiments confirm that single-stranded DNA is bound under physiological conditions and in an extended conformation (12). Cross-linking experiments demonstrate that at least four 3′-terminal nucleotides must be separated from the template strand in order for KF to excise the terminal nucleotide and that a two nucleotide strand separation is required for T4 DNA polymerase (13). The mechanism, however, for converting the natural substrate for proofreading, a duplex DNA with a terminal mismatch, into a DNA with a strand separation of two or more nucleotides has not been elucidated.

We report here the biochemical characterization of a mutant T4 DNA polymerase with an apparent defect in strand separation. This mutant, the G255S-DNA polymerase (Gly to Ser substitution at codon 255), was isolated by two different genetic selection strategies for mutant DNA polymerases with reduced exonucleolytic proofreading. In one method, the mutant was identified on the basis of a strong mutator phenotype (2). In the second method, mutations that encode G255A (Ala substitution for Gly-255) and G255S amino acid substitutions were identified repeatedly as suppressors of the excessive proofreading produced by other DNA polymerase mutations (9). Residue Gly-255 and the surrounding region has been identified as a “hot spot” by our genetic selection methods for mutations that decrease proofreading (9). Although it has only been assumed that Gly-255 resides in the exonuclease domain based on protein sequence comparisons to KF (2–5), recent structural studies of an amino-terminal fragment of the T4 DNA polymerase verify that residue Gly-255 is located in the exonuclease domain (14).

Because of the numerous independent genetic isolations of

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‡Scientist of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed. Tel.: 403-492-5383; Fax: 403-492-9234; E-mail: lehua@gpu.srv.ualberta.ca.

The abbreviations used are: KF, Klenow fragment; 2AP, 2-aminopurine; d2APMP, deoxy-2-aminopurine nucleoside monophosphate; DTT, dithiothreitol.
the G255S-DNA polymerase, this mutant enzyme was purified and the exonuclease activity measured. Surprisingly, the wild type level of 3′ → 5′-exonuclease activity was detected with single-stranded DNA substrates which indicates that the ability to hydrolyze a phosphodiester bond is not affected in the mutant enzyme; however, significantly reduced activity was observed with double-stranded DNAs (9). These results suggest that some aspect in the process of converting duplex DNA with a mispaired primer terminus, the natural substrate for proof-reading, into the more strand-separated DNA required for 3′ → 5′-exonuclease activity is defective in the mutant. Thus, determination of the biochemical basis of this defect in proofreading by the G255S-DNA polymerase may reveal insights into the mechanism used by DNA polymerases to achieve strand separation.

We have previously used the fluorescent analogue of adenine, 2-aminopurine (2AP), positioned in a terminal base pair, to study the pre-steady state kinetics of the 3′ → 5′-exonuclease activity of T4 DNA polymerase (15). In order to investigate the strand separation step, we designed a series of DNA substrates with an increasing number of mispairs at the primer terminus in order to simulate varying degrees of strand separation.

By employing rapid mixing, stopped-flow techniques with sensitive fluorescence detection, we were able to investigate the pre-steady state kinetics of the 3′ → 5′-exonuclease reaction of T4 wild type- and G255S-DNA polymerases within the millisecond time scale. Similar levels of exonuclease activity were detected for the two enzymes with single-stranded DNA substrates, but excision rates were significantly reduced for the mutant on duplex DNA substrates with preformed strand separations of less than two nucleotides. Another difference between the mutant and wild type enzymes was that while A-T base pairs upstream of the 2AP mismatch, compared with upstream G-C base pairs, stimulated excision rates by the wild type T4 DNA polymerase, the A + T or G + C richness of the flanking DNA did not affect excision rates by the G255S-DNA polymerase. A + T-rich sequences are expected to facilitate strand separation step, we designed a series of DNA substrates with an increasing number of mispairs at the primer terminus in order to simulate varying degrees of strand separation.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Enzymes**—Wild type T4 DNA polymerase and the G255S-DNA polymerase were produced from the T4 DNA polymerase expression vector (9, 18) and purified as described previously (19).

**DNA Substrates**—Oligonucleotides were prepared using standard β-cyanoethyl phosphoramidite reagents from Applied Biosystems. 2AP was incorporated at the 3′-end using a 2AP-derivatized controlled pore glass solid support (20, 21). The oligonucleotides were purified by polyacrylamide gel electrophoresis and then dialyzed against distilled water with an increasing concentration of DTT. The primer-template duplexes were annealed in buffer containing 25 mM HEPES, pH 7.5, and 50 mM NaCl as described (15). The concentration of the template strand was 20% higher than the concentration of the 2AP primer strand to ensure that all of the primer was annealed (15). If any unannealed primer was present in the reaction, then a burst of hydrolysis due to rapid degradation of single-stranded DNA would be detected; this was not observed.

The primer-template used are described in Table I. Two sets of DNA duplexes (17/30-mers) were prepared; one set was relatively more G + C-rich upstream of the terminal 2AP base pair and one set was more A + T-rich. For the G + C-rich set of primer-templates, 2AP was paired opposite either template T, A, C, or G (XZ1-XZ4). For the A + T-rich set of primer-templates, 2AP was paired opposite template T (XZ8) or C (XZ8). For both the G + C-rich and A + T-rich primer-templates, an A-T base pair was immediately adjacent to the 2AP base pair. The next three upstream base pairs were then either consecutive G-C base pairs (XZ12 primer set) or A-T base pairs (XZ13 primer set). Another variation was to introduce one or two mispairs upstream of the 2AP-C base pair. To produce two 3′-terminal mispairs, the terminal 2AP-C mispair was adjacent to an upstream T-C mispair (template XZ6) for the G + C-rich primer set and template XZ10 for the A + T-rich primer set. To produce three terminal mispairs, the terminal 2AP-C mispair was adjacent to the T-C mispair, and this mispair was adjacent to an upstream G-G mispair in the G + C-rich primer set (template XZ7) and to an A-G mispair in the A + T-rich primer set (template XZ11).

**Methods**

Absorbance measurements to determine protein and DNA concentrations were carried out with a DU650 Beckman spectrophotometer equipped with a thermally jacketed 1-cm cuvette holder. Enzyme concentrations were determined by absorbance measurements at 280 nm using the experimentally determined extinction coefficient of 1.492 × 10^4 M^-1 cm^-1 (3). DNA concentrations were determined from absorbance measurements at 260 nm. Extinction coefficients were calculated based on the nucleotide composition of the oligonucleotides using the EXTINCT.FOR program developed by J. M. Beechem, Vanderbilt University (based on Ref. 22).

**Steady state fluorescence spectra** were collected by exciting the samples at 310 nm and scanning from 330 to 480 nm using a monochromator. The excitation monochromator was set at a slit width of 3 nm and the emission monochromator was set at 5 nm. The photomultiplier voltage was set at 999 V. Background emissions from buffer were subtracted from all scans.

Stopped-flow emission scans and kinetic measurements were performed using the SX.17MV spectrofluorimeter from Applied Biophysics. Pre-steady state kinetic experiments were performed in the stopped-flow using a 335-nm cut-off filter and 310 nm excitation light from a xenon arc source passing through a 2-nm band pass. The photomultiplier voltage was set at 700 V, and scattered light was subtracted from the reaction signal by applying an offset voltage. All kinetic measurements were carried out at 20.0 ± 0.5°C by allowing water from a thermostated bath to flow around the optical cell and the reservoir syringes.

Excision reactions were initiated in the stopped-flow apparatus by mixing equal volumes of T4 DNA polymerase and MgCl2 with the DNA primer-templates. One syringe contained 1.4 μM T4 DNA polymerase, 2 mM DTT, 16 mM MgCl2, 25 mM HEPES, pH 7.5, and 50 mM NaCl. The second syringe contained 40 μM DNA (DNA concentrations were expressed in terms of 3′-2AP ends), 0.5 mM EDTA, 25 mM HEPES, pH 7.5, and 50 mM NaCl. Equal volumes (approximately 50 μl) of each solution were mixed within the instrument dead time of 1.5 ms by pneumatic rams triggered by 140 p.s.i. of N2 gas. Final concentrations of reaction components were 700 nM T4 polymerase, 200 nM DNA, 1 mM DTT, 8 mM MgCl2, 25 mM HEPES, pH 7.5, and 50 mM NaCl. Excision reactions measured in the presence of dNTPs (Pharmacia Biotech Inc.) were done by adding dATP, dCTP, dGTP, and dTTP, each at 200 μM, to the syringe with enzyme. After mixing with the DNA solution, the final dNTP concentrations were 100 μM.

Pre-steady state excision rates were measured from the increase in fluorescence intensity with time due to the release of d2APMP positioned at the 3′-terminus of the preformed DNA substrates. 2AP in DNA is quenched, but d2APMP in solution is highly fluorescent. Curves were fit either to single (monophasic) or double (biphasic) exponential equations. Between six and eight determinations were performed for each reaction and mean values were calculated.

**RESULTS**

**Wild Type T4 DNA Polymerase**—One objective of our studies was to investigate the effect of base pair stability and local DNA sequence on the T4 DNA polymerase 3′ → 5′-exonuclease activity. 2AP was paired opposite the four template bases, T, A, C, or G, with either upstream A-T or G-C base pairs. We observed, as also reported by Bloom et al. (15), that hydrolysis rates were dependent on the nature of the 3′-terminal base pair. The slowest rate was observed for the 2AP-A base pair, an even faster rate for the 2AP-C base pair, and the fastest rate was observed for the
2AP-G base pair, the most “incorrect” base pair. The same order for removal of d2APMP from 2AP paired opposite the four template bases was observed in another sequence context (15). The efficiency of excision correlates with the stability of the 2AP-X base pairs as predicted from $T_m$ measurements, which increase in the order of 2AP-G < 2AP-C < 2AP-A < 2AP-T (21).

The kinetic traces for the hydrolysis reactions with the 2AP-T and 2AP-A DNA substrates appeared to be biphasic. The best curve fits for the experimental traces were achieved by using a double exponential equation. The curve fit for the G + C-rich, 2AP-T DNA substrate is shown in Fig. 1, panel B. Monophasic kinetics was observed for reactions with the 2AP-C and 2AP-G DNA substrates. The curve fit for the G + C-rich, 2AP-C DNA substrate, described by a single exponential equation, is shown in Fig. 1, panel C. The 2AP-T and 2AP-C DNA substrates were selected for further study because of the respective biphasic and monophasic kinetics observed for the hydrolysis reactions for the two substrates, and because of the relevance of proofreading 2AP-T and 2AP-C primer termini to understanding how 2AP promotes base substitution mutagenesis.

The pre-steady state excision rates for the G + C-rich 2AP-T and 2AP-C DNA substrates, calculated from the kinetic traces shown in Fig. 1, are given in Table II. The 2AP-T DNA was degraded more slowly than the 2AP-C DNA due to the major contribution of the slower phase, described by rate constant $k_2$, to the overall reaction rate. The faster phase for degradation of the 2AP-T DNA, described by rate constant $k_1$, was similar in magnitude to the hydrolysis rate measured for removal of d2APMP from the G + C-rich 2AP-C DNA substrate (compare 17 to 20 s$^{-1}$).

Excision rates were also determined for the 2AP-T and 2AP-C base pairs in an A + T-rich local environment. These DNA substrates have four consecutive A-T base pairs upstream of the reporter 2AP base pair (Table I). Biphasic kinetics was again observed for the 2AP-T DNA, but each phase was approximately 2-fold faster than observed for the G + C-rich DNAs (Table II). A single phase was observed for the A + T-rich 2AP-C DNA substrate, and excision was also 2-fold faster than detected for the G + C-rich 2AP-C DNA. As observed with the G + C-rich 2AP-T DNA substrates, the rate for removal of d2APMP from the 2AP-C DNA substrate (about 42 s$^{-1}$) was similar to the faster hydrolysis rate, described by $k_1$ (about 37 s$^{-1}$), for hydrolysis of the A + T-rich AP-T DNA substrate. Thus, the rate for the faster of the two hydrolysis reactions observed for the 2AP-T DNA substrates resembles the single reaction rate detected for the 2AP-C DNA substrates.

Biphasic kinetics has been observed previously for the proofreading reaction by T4 DNA polymerase. In a rapid quench assay, a burst of hydrolysis was observed for the excision of a terminal mismatched nucleotide (23). The slow and fast phases were interpreted to indicate partitioning of the DNA between two states: one that is initially inactive for hydrolytic cleavage and a second that is pre-activated for hydrolytic cleavage. These states may correspond to DNA bound initially in either the polymerase (inactive) or exonuclease (activated) active centers (15). Thus, the faster phase, $k_1$, in reactions with the 2AP-T DNA, may describe removal of d2APMP from DNA bound in an activated or partially activated state for the hydrolysis reaction, whereas the slower phase, described by rate constant $k_2$, may describe the hydrolysis rate for DNA first.
Primer 1 and 2 differ in the nucleotide triplet (bold) upstream of the 3’ terminus, i.e. primer 1 is G+C-rich while primer 2 is A+T-rich. Templates XZ2–XZ7 are identical to template XZ1 except for the underlined nucleotides. Templates XZ9–XZ11 are identical to template XZ8 except for the underlined nucleotides.

**Primers**

Primer 1, XZ12 (G + C-rich)

Templates

XZ1

XZ2

XZ3

XZ4

XZ6

XZ7

Primer 2, XZ13 (A + T-rich)

Templates

XZ5

XZ9

XZ10

XZ11

**Table I**

| No. of mispairs | Description of 3’-mispairs | G + C-rich DNA substrates | A + T-rich DNA substrates |
|-----------------|-----------------------------|---------------------------|--------------------------|
|                 |                             | $k_1$                     | $a_1$                    | $k_2$ | $a_2$ |
| 0               | 2AP-T                       | 17.2 ± 2.8                | (0.2)                    | 3.9 ± 0.2 | (0.8) |
|                 | 2AP-C                       | 19.9 ± 0.3                |                          | 42.0 ± 2.1 |
| 2               | T-C                         | 87.8 ± 1.4                |                          | 156.5 ± 7.1 |
| 3               | A/G-G                       | 157.2 ± 3.8               |                          | 202.2 ± 12.4 |
|                 | Single-stranded DNA         | 175.7 ± 5.7               |                          | 228.4 ± 13 |

*The complete sequences of the DNA substrates are given in Table I. The 2AP-T base pair is the most correct base pair used in this study and is given the “0” mispair designation, although the 2AP-T base pair is less stable than the A-T base pair (35). The single mispair DNA has a terminal 2AP-C, and the two mispair DNA has the terminal 2AP-C mispair and an adjacent T-C mispair. Three terminal mispairs were produced by the addition of a G-G mispair adjacent to the T-C mispair in the G + C-rich DNA set and an A-G mispair in the A + T-rich DNA set.

**Table II**

| No. of mispairs | Description of 3’-mispairs | G + C-rich DNA substrates | A + T-rich DNA substrates |
|-----------------|-----------------------------|---------------------------|--------------------------|
|                 |                             | $k_1$                     | $a_1$                    | $k_2$ | $a_2$ |
| 0               | 2AP-T                       | 17.2 ± 2.8                | (0.2)                    | 3.9 ± 0.2 | (0.8) |
|                 | 2AP-C                       | 19.9 ± 0.3                |                          | 42.0 ± 2.1 |
| 2               | T-C                         | 87.8 ± 1.4                |                          | 156.5 ± 7.1 |
| 3               | A/G-G                       | 157.2 ± 3.8               |                          | 202.2 ± 12.4 |
|                 | Single-stranded DNA         | 175.7 ± 5.7               |                          | 228.4 ± 13 |

*The rate constants for the two phases are given by $k_1$ and $k_2$, respectively.

The amplitudes of the two phases of the biphasic time courses are given by $a_1$ and $a_2$, respectively.

bound in the polymerase active center and then transferred to the exonuclease active center. In experiments described below, we provide evidence that the 2AP-T DNA is indeed partitioned between two states.

Additional steps in strand separation were probed by measuring excision rates for DNAs with one and two additional preformed mispairs adjacent to the terminal 2AP-C mispair. Fluorescence of 2AP in DNA, although quenched, can be used to verify the extent of strand separation at the primer terminus since 2AP fluorescence is quenched more in double- than in single-stranded DNA. Fluorescence intensity was lowest for the 2AP-T primer-template and increased as the number of mispairs at the primer terminus increased (Fig. 2). Fluorescence intensity was highest for the single-stranded 2AP primer.

Single-phase kinetics was observed for primer-templates with one or more adjacent mispairs. The time course for the primer-template, which has a terminal 2AP-C mispair and an adjacent T-C mispair, is shown in Fig. 3. Excision rates increased with the degree of preformed strand separation; substrates with three mispairs produced the highest rates and approached the rates measured for the single-stranded DNAs (Table II). A + T-richness of the upstream base pairs stimulated the removal of the terminal d2APMP from the mispaired DNAs as observed with the 2AP-T and 2AP-C DNAs.

Because the excision rates for DNA substrates with two and three preformed mispairs were faster than the excision rates detected for removal of d2APMP from the 2AP-T and 2AP-C DNA substrates, we propose the existence of a pre-exonuclease complex that requires further processing to form the complex that is fully activated for the hydrolysis reaction. Association rates for forming the pre-exonuclease state and for binding DNA in the polymerase active center are assumed to be much more rapid than the reaction steps described by $k_1$ and $k_2$. The data obtained with the 2AP-T and preformed strand-separated DNA substrates are consistent with the reaction steps summa-
FIG. 3. Time course and curve fit for the reaction between wild type T4 DNA polymerase and the primer-template with two terminal mispairs (T-C adjacent to terminal 2AP- C) with upstream G-C base pairs. The reaction was initiated as described in Fig. 1. The experimental data were fit to a single exponential equation which gives a more random distribution in the residuals compared with a double exponential equation.

The fully activated (enzymeexo)* 2AP-T DNA* complex can be formed from two starting points in our reaction conditions. In one starting point, the 2AP-T DNA is bound initially in the polymerase active center (enzymepol 1)* 2AP-T DNA. In the second pathway, the 2AP-T DNA is bound in a pre-exonuclease state (enzyme pre-exo)* 2AP-T DNA. The pre-exonuclease state requires further processing for conversion to the activated state (enzyme enz)* 2AP-T DNA). DNA bound initially in the polymerase active center may be transferred in an intramolecular reaction to form the pre-exonuclease state which is then further processed to form the activated complex. Formation of the pre-exonuclease state and the activated complex are rate-limiting steps, and the rates of formation are dependent on the local sequence.

Since excision rates for DNA substrates with three preformed mispairs were nearly equal to rates measured for the single-stranded DNA substrates, these DNAs did not require significantly further preparation for the excision reaction and were bound directly from solution to form the activated complex. We infer from these results that at least three nucleotides at the primer terminus must be separated from the template strand in order to form the activated complex since at least three preformed mispairs were required in order for the excision rates for duplex DNAs to match the excision rates detected for the hydrolysis of single-stranded DNAs. The rates reported here for removal of d2APMP from single-stranded DNA (175–230 s⁻¹) are in the same range but faster than the hydrolysis rate reported for degradation of single-stranded by the rapid quench assay (100 s⁻¹) (23). The hydrolysis rate for single-stranded DNA may be limited by the rate of association of the enzyme with the DNA substrate.

G255S-DNA Polymerase—A second objective of our studies was to investigate the strand separation step of the excision reaction by determining the biochemical basis of the exonuclease deficiency of the G255S-DNA polymerase. The same experiments reported for the wild type T4 DNA polymerase were performed with the G255S-DNA polymerase. The mutant DNA polymerase exhibited close to the wild type level of 3’ → 5’-exonuclease activity on single-stranded DNA (compare Tables II and III); however, much slower rates were observed with the 2AP-T and 2AP-C DNA substrates. As observed for wild type T4 DNA polymerase, the G255S-DNA polymerase produced a biphasic trace for the 2AP-T substrate. While the rate, k₁, for the faster phase of the G255S-DNA polymerase reaction was similar to the k₁ measured for the wild type T4 DNA polymerase (14.9 compared with 17.2 s⁻¹, G + C-rich DNA), the phase described by the rate constant k₂ was more than 10-fold slower for the mutant DNA polymerase (0.34 versus 3.9 s⁻¹). In contrast to the kinetics observed for the wild type T4 DNA polymerase, biphasic kinetics was also observed for the 2AP-C DNA substrate (Table III). The rates for the mutant for the faster reaction phase described by k₁ were similar for the 2AP-T and 2AP-C DNAs, but the slower phase, k₂, was about 4-fold faster for the 2AP-C DNA compared to the AP-T DNA.

Another difference in exonuclease activity between the wild type and mutant DNA polymerases was that the excision rates of the G255S-DNA polymerase were not significantly affected by upstream A-T or G-C base pairs (Table III). It has been proposed that high A + T content of the primer terminus compared with high G + C content increases excision rates by assisting the strand separation process (16, 17, reviewed in Ref. 24). We observed consistently 2-fold higher excision rates by the wild type T4 DNA polymerase for the A + T-rich set of substrates compared with the G + C-rich DNAs (Table II), but since A + T-richness did not stimulate excision rates by the G255S-DNA polymerase, the mutant apparently could not take advantage of A + T-richness to prepare duplex DNA for the hydrolysis reaction.

Slower excision rates were also observed for the G255S-DNA polymerase compared with the wild type enzyme with the DNA substrates containing two and three preformed terminal mispairs (Table III). Unlike the wild type DNA polymerase that displayed faster excision rates for the DNA substrates with three mispairs compared with the DNAs with two mispairs, similar excision rates were detected for the mutant with both types of substrates. Excision rates for the DNAs with three mispairs by the mutant were also 2–3-fold slower than the rates detected for degradation of single-stranded DNAs. Thus, when DNAs with three preformed terminal mispairs were degraded by the wild type enzyme at nearly the same rates observed for degradation of single-stranded DNAs (Table II), the slower excision rates for the G255S-DNA polymerase suggest that the mutant enzyme requires additional strand separation in order to achieve the optimal excision rates that were observed for the single-stranded DNA substrates.

Scheme I, proposed for the wild type T4 DNA polymerase, can also be used to summarize the data for the G255S-DNA polymerase if the rate constants k₁ and k₂ are adjusted downward (Table III). The reaction step that is most defective for the mutant DNA polymerase was in formation of the pre-exonuclease complex described by rate constant k₂. The reaction pathway described by k₂ simulates the in vivo proofreading reaction since during chromosome replication the DNA primer normally
resides in the polymerase active center and is transferred to the exonuclease active center after a nucleotide has been misincorporated. The strong mutator phenotype observed for the G255S-DNA polymerase is consistent with the proposal that the reaction described by rate constant $k_2$, which is $\approx 10$-fold slower for the mutant compared to the wild type T4 DNA polymerase, reflects the decreased ability of the mutant DNA polymerase to proofread a mismatched base pair in vivo. Since the G255S-DNA polymerase can hydrolyze single-stranded DNA at rates similar to those detected for the wild type enzyme, the exonuclease active center of the mutant appears to be intact and fully functional. The mutant is defective, however, in processes that convert duplex DNA into the strand-separated DNA substrate required for proofreading.

Excision/Incorporation Competition Experiments—A key component of the model presented in Scheme I is that the 2AP-T DNA can be bound into the polymerase active center. The location of the primer in either the polymerase or exonuclease active centers can be determined in reactions that also include dNTPs. Proofreading during chromosome replication is in competition with primer elongation. The 3’-primer end of a DNA duplex is extended or proofread depending on the status of the primer terminus. If the primer terminus has a correct base pair, extension will be the favored reaction. If the base pair at the primer terminus is incorrect, proofreading will be the favored reaction since extension of a mispaired primer terminus is significantly slower than extension of a correct terminal base pair (reviewed in Ref. 24).

We modified the fluorescent assay developed to measure pre-steady state excision rates to also detect extension of the 2AP-primer. Nucleotides were added to the syringe with enzyme and $\text{Mg}^{2+}$ while DNA substrates were in the second syringe. Rapid mixing of the two solutions allowed formation of the enzyme-DNA-dNTP ternary complex. If the complex is activated for nucleotide insertion and replication proceeds, the excision reaction will be prevented. Thus, in comparison to reactions without dNTPs, there will be reduced formation of the fluorescent d2APMP product from the enzyme-DNA-dNTP complex if the primer is extended. The amount of d2APMP produced in the absence of dNTPs less the amount of d2APMP produced in the presence of dNTPs will indicate the amount of 2AP-primer extended without first removing the terminal d2APMP.

Excision-extension competition reactions were done with the 2AP-T DNA substrate. The most striking difference observed in the presence of dNTPs compared with reactions without dNTPs was the reduced formation of d2APMP, only about 30–40% of the d2APMP was excised in reactions with dNTPs (Fig. 4) while all of the d2APMP was removed in reactions without dNTPs (Fig. 1). The reactions with the wild type enzyme and the 2AP-T DNA substrates in the presence of dNTPs were biphasic as observed for the reactions in the absence of dNTPs, and similar reaction rates were detected. For the slower reaction phase, described by rate constant $k_{3b}$, the rates measured were $4.5 \pm 1.5 \text{ s}^{-1}$ for the G + C-rich DNA and $11.2 \pm 5.5 \text{ s}^{-1}$ for the A + T-rich DNA which are similar to the rates measured in the absence of dNTPs (compare to Table II); however, in contrast to the reactions without dNTPs, the contribution of this slower phase to the overall excision reaction was reduced from 80% observed in the absence of dNTPs to 20% as expected if a significant population of the DNA molecules bound in the polymerase active center were extended rather than proofread. More d2APMP was produced in reactions with the A + T-rich 2AP-T DNA than with the G + C-rich 2AP-T DNA indicating that the extendability of the 2AP-T base pair was reduced by a local A + T-rich environment, which is consistent with previous findings (16). For the 2AP-T DNA bound in the polymerase active center in the presence of dNTPs, extension was favored over excision which suggests that the formation of the pre-exonuclease complex, described by rate constant $k_{3a}$, may be a kinetic barrier (23) that protects correctly base paired primer termini from degradation.

Even less d2APMP was produced in reactions with dNTPs by the G255S-DNA polymerase (Fig. 4). We attempted to determine rate constants for the small amount of d2APMP produced. The reaction appeared to be monophasic with a rate constant of $9.0 \pm 0.7 \text{ s}^{-1}$ for the G + C-rich DNA and $13.9 \pm 0.3 \text{ s}^{-1}$ for the A + T-rich DNA. These values are similar to the rate constant $k_1$ determined in reactions without dNTPs. Since no d2APMP was formed with the slower rate constant $k_{3b}$ in the presence of dNTPs, all of the 2AP-T DNA bound in the polymerase active center by the mutant DNA polymerase appeared to be ex-
the fluorescent d2APMP to further probe the molecular details of polymerase, and a sensitive assay employing the release of mutationally distinct mutant T4 DNA polymerase, the G255S-DNA replication (25–27). In this study, we have used a functional incorporation and is thus a major determinant of the fidelity of DNA polymerases corrects mistakes in nucleotide incorporation (Fig. 5). In reactions with preformed mismatches at the partially single-stranded DNA required for the hydrolysis reaction. Excision rates slower than the rates observed for mismatched primer termini, proofreading is prevented. Thus, although the G255S-DNA polymerase can effectively hydrolyze single-stranded DNA, we propose that formation of the pre-exonuclease complex by the mutant DNA polymerase is a kinetic barrier to proofreading some mismatched primer termini and is the basis of the mutator phenotype observed in vivo. This proposal is supported by the greater ability of the G255S-DNA polymerase, compared with the wild type T4 DNA polymerase, to extend the 2AP-T base pair (Fig. 4).

The DNA in the pre-exonuclease state is not fully prepared for the excision reaction, since faster excision rates were observed for DNAs with two and three preformed terminal mispairs and for single-stranded DNAs (Tables II and III). The natural substrate for DNA polymerase proofreading is a DNA with a mismatched primer terminus. DNA with a single terminal mispair must be denatured further in order to be a substrate for the hydrolysis reaction catalyzed by residues in the exonuclease active center of DNA polymerases. Single-stranded DNA has been detected in the exonuclease active centers of KF and T4 DNA polymerases by x-ray crystallography (11, 14, 28) and by observing the fluorescence decay characteristics of 2AP-DNA when bound in the exonuclease active center of KF (12). The single-stranded nature of the DNA substrate in the exonuclease active center has also been inferred from numerous enzymatic studies which demonstrate that reaction conditions that stimulate strand separation also increase exonuclease activity (16, 17, 25, 26).

How much strand separation is required for the hydrolysis reaction? Cross-linking experiments indicate that at least two nucleotides must be separated in order for the T4 DNA polymerase to remove the 3’-terminal nucleotide (13). In studies that used streptavidin bound to biotin located at various nucleotide positions in the DNA template, the polymerase and exonuclease active centers were determined to be separated by two to three nucleotides (29). Our results indicate that a strand separation of three nucleotides is required for optimal hydrolysis rates, but a strand separation of two nucleotides may be an intermediate in the process. For the wild type T4 DNA polymerase and the G + C-rich DNA, the addition of a second preformed mispair to the 2AP-C DNA stimulated excision rates about 4-fold, but the addition of a third mispair increased rates just 2-fold, and a smaller stimulation by addition of a third

FIG. 5. Cartoon depicting 2AP-T DNA in the polymerase active center, in the pre-exonuclease state, and in the exonuclease active center in a state fully activated for the hydrolysis reaction. Rate constants $k_1$ and $k_2$ are described in the text and in Scheme I.

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The small amount of d2APMP produced was from DNA bound directly in the pre-exonuclease complex. If $k_2$ is a kinetic barrier to the proofreading reaction as proposed, this barrier is higher for the G255S-DNA polymerase and may account for the mutator phenotype observed in vivo.

DISCUSSION

DNA polymerase proofreading has been the subject of intense study since the discovery that the 3’ → 5’-exonuclease activity of DNA polymerases corrects mistakes in nucleotide incorporation and is thus a major determinant of the fidelity of DNA replication (25–27). In this study, we have used a functionally distinct mutant T4 DNA polymerase, the G255S-DNA polymerase, and a sensitive assay employing the release of fluorescent d2APMP to further probe the molecular details of the 3’ → 5’-exonuclease activity of T4 DNA polymerase. The main objective of our study was to learn more about how DNA with a single terminal mismatch, the product of a nucleotide misincorporation event, is converted into a substrate for the hydrolysis reaction. Excision rates slower than the rates observed for DNAs with three mispairs or for single-stranded DNA indicate that one or more rate-limiting steps are required to prepare the DNA for the excision reaction.

Our results suggest a proofreading pathway in which DNA is first transferred from the polymerase active center to a pre-exonuclease complex and then further processed to produce the partially single-stranded DNA required for the hydrolysis reaction (Fig. 5). In reactions with preformed mismatches at the primer terminus, DNA was bound directly from solution to form the pre-exonuclease complex or into the polymerase active center (Scheme I). DNA bound directly to form the pre-exonuclease complex was the predominant reaction with DNA substrates with more than one terminal mispair. Formation of the pre-exonuclease complex from DNA bound initially in the polymerase active center is described by rate constant $k_2$ (Scheme I and Fig. 5). This reaction likely requires a conformation change since the rate constant $k_2$ for the wild type enzyme was sensitive to the A + T or G + C richness of upstream base pairs which suggests that some strand separation may be required in the formation of the pre-exonuclease complex. We also envision that the primer terminus is moved at least partially out of the polymerase active center (Fig. 5) because a residue in the exonuclease domain, Gly-255, is implicated as a participant in this reaction step. The most significant difference between the wild type and G255S-DNA polymerases was in formation of the pre-exonuclease complex by the reaction step described by $k_2$. The rate of formation of the pre-exonuclease complex, which is much slower than the hydrolysis reaction, may serve as a kinetic barrier to proofreading some correct primer terminus. If the rate of formation of the pre-exonuclease complex, however, is reduced significantly, as observed for the G255S-DNA polymerase, then the kinetic barrier may be too high so that even for mismatched primer termini, proofreading is prevented. Thus, although the G255S-DNA polymerase can effectively hydrolyze single-stranded DNA, we propose that formation of the pre-exonuclease complex by the mutant DNA polymerase is a kinetic barrier to proofreading some mismatched primer termini and is the basis of the mutator phenotype observed in vivo. This proposal is supported by the greater ability of the G255S-DNA polymerase, compared with the wild type T4 DNA polymerase, to extend the 2AP-T base pair (Fig. 4).

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mispair was observed for the A + T-rich DNA (Table II). Studies with the G255S-DNA polymerase indicate clearly that a strand separation of two nucleotides is a critical step (Table III). Although reaction rates detected for the mutant were lower than the excision rates detected for the wild type enzyme with DNA substrates containing two or three preformed terminal mispairs, the mutant was considerably more active on these substrates than with the more base paired 2AP-T and 2AP-C DNA substrates.

What is the mechanism of strand separation? One component of strand separation is the DNA itself, specifically, the physical structures that the primer terminus can assume. The primer-terminal region is in equilibrium between various partially annealed and denatured states. Intrinsic primer terminus instability, however, is unlikely to be sufficient to promote the extent of denaturation that is required for the hydrolysis reaction. In the case of the T4 DNA polymerase, for example, fully duplex DNA can be degraded by the 3’ → 5’-exonuclease activity in the absence of dNTPs, and some correct primer termini are even proofread during chromosome replication, particularly by “antimutator” DNA polymerases (reviewed in Refs. 24 and 30). Thus, we propose a second component in the strand separation process, namely that the DNA polymerase actively participates in separating the primer from the template strand. Evidence that the DNA polymerase assists in the strand separation process is provided by the G255S-DNA polymerase. Although the mutant has lower activity on duplex DNA substrates, the residual activity was sufficient to allow examination of the role of DNA “breathing” in the strand separation process. If the DNA is a major determinant of strand separation, then the wild type DNA polymerase and the mutant alike should have faster excision rates with A + T-rich DNA substrates compared with G + C-rich DNAs. Faster rates were observed for the wild type T4 DNA polymerase but not for the G255S-DNA polymerase (Tables II and III). Since the excision reaction by the mutant enzyme was not stimulated by A + T-richness, “frayed ends” or “breathing” were not sufficient to denature the primer for proofreading.

Different mechanisms to achieve DNA polymerase-mediated strand separation can be envisioned. Certain amino acid residues may bind the primer terminus and via a conformational change pull the strands apart. Another possibility is that the DNA polymerase may exploit DNA breathing at the primer end by preventing strand renaturation. For example, a DNA polymerase structure may be used as a wedge so that when this structure is inserted between the template and primer strands the equilibrium between base pairing and denaturation is driven toward strand separation. These models cannot be distinguished from our studies; however, we can suggest a structural element that may participate in the strand separation process. The crystal structure of an amino-terminal fragment of the T4 DNA polymerase has been determined recently, and two regions were identified that are present in T4 DNA polymerase but missing in KF (14). One of these regions is a loop structure that is formed by residues 240 and 265 (Fig. 6). This region includes Gly-255, the target of the mutational analysis. It is likely that substituting glycine, a “turning point” in the loop, with serine would affect the structure and may prevent the loop structure or residues in the loop in assisting strand separation.

A long-standing question in DNA polymerase proofreading has been the reason for the high 3’ → 5’-exonuclease activity of the T4 DNA polymerase compared with E. coli DNA polymerase I. The KF excises a terminal mismatched nucleotide about 1000-fold more slowly than T4 DNA polymerase. Kuchta et al. (31) report an excision rate of $3.4 \times 10^{-3}$ s$^{-1}$ for KF for removal of a dTMP opposite template T, whereas Capson et al. (23) and we have observed excision rates of 4–5 s$^{-1}$ for T4 DNA polymerase. One possibility for the difference in 3’ → 5’-exonuclease activities between the two DNA polymerases is that KF does not have as an efficient mechanism for strand separation. Structural studies indicate that KF does not have the Gly-255 loop structure. In support of this hypothesis is the possibility that the loop structure may be conserved in other proofreading DNA polymerases that function in chromosome replication. T4 DNA polymerase is a functional homologue of the human DNA polymerase δ (32). By slightly altering the protein sequence alignments of DNA polymerase sequences proposed by Braithwaite and Ito (33), residues analogous to Gly-255 in T4 DNA polymerase may correspond to Gly-442 in the human DNA polymerase δ and to Gly-447 in the yeast DNA polymerase δ (9).

If certain amino acid residues assist in separating the primer strand from the template strand, as we propose, then other residues must function to limit the hydrolysis reaction so that just the 3’-terminal nucleotide is excised. A critical feature of DNA polymerase proofreading is that just the terminal incorrect nucleotide is removed (25, 26). Further degradation would interfere with chromosome replication and waste dNTPs. While two successive cleavage reactions have been detected with a DNA substrate with three preformed mispairs, the third unpaired nucleotide could not be removed processively (34). Excision may be restricted to the terminal one or two nucleotides because of specific interactions between certain amino acid residues and the single-stranded DNA in the exonuclease active center.

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