Interaction of DNA Polymerase I (Klenow Fragment) with DNA Substrates Containing Extrahelical Bases: Implications for Proofreading of Frameshift Errors during DNA Synthesis†

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ABSTRACT: Frameshift mutagenesis occurs through the misalignment of primer and template strands during DNA synthesis and involves DNA intermediates that contain one or more extrahelical bases in either strand of the DNA substrate. To investigate whether these DNA structures are recognized by the proofreading apparatus of DNA polymerases, time-resolved fluorescence spectroscopy was used to examine the interaction between the Klenow fragment of DNA polymerase I and synthetic DNA primer−templates containing extrahelical bases at defined positions within the template strand. A dansyl probe attached to the DNA was used to measure the fractional occupancies of the polymerase and 3′−5′ exonuclease sites of the enzyme for DNA substrates with and without the extrahelical bases. The presence of an extrahelical base at the first position from the primer 3′ terminus increased the level of partitioning of the DNA substrates into the 3′−5′ exonuclease site by 3−7-fold, relative to the perfectly base-paired primer−template, depending on the identity of the extrahelical base. The ability of different extrahelical bases to promote partitioning of DNA into the 3′−5′ exonuclease site decreased in the following order: G > A ≈ T > C. The results of partitioning measurements for DNA substrates containing a bulged adenine base at different positions within the template showed that an extrahelical base is recognized up to five bases from the primer 3′ terminus. The largest effects were observed for the extrahelical base at the third or fourth positions from the primer terminus, which increased the level of partitioning of DNA into the 3′−5′ exonuclease site by 8- and 18-fold, respectively, relative to that of the perfectly base-paired substrate. Steady-state fluorescence measurements of analogous primer−templates containing 2-aminopurine (AP) at the primer 3′ terminus indicate that extrahelical bases increase the degree of terminus unwinding, especially when close to the terminus. In addition, steady-state kinetic measurements of removal of AP from the primer−templates indicate that the exonucleolytic cleavage activity of Klenow fragment is correlated with the increased level of partitioning of bulged DNA substrates to the 3′−5′ exonuclease site relative to that of properly base-paired DNA. The results of this study indicate that misalignment of primer and template strands to generate an extrahelical base strongly promotes transfer of a DNA substrate to the 3′−5′ exonuclease site, suggesting that the premutational intermediates in frameshift mutagenesis are subject to proofreading by the polymerase.

In addition to base substitution errors, genetic information can also be corrupted by the addition or deletion of nucleotides during DNA replication (*1*, *2*). These frameshift errors arise via misalignment of the primer and template strands during polymerization by DNA polymerases and occur preferentially in homopolymeric and other repetitive sequences (*3*, *4*). Frameshift error rates are lower for DNA polymerases having an associated 3′−5′ exonuclease activity, indicating that the misaligned DNA intermediates can be corrected by exonucleolytic proofreading. However, very little is known about how the polymerase is able to recognize and correct these misaligned DNA structures.

To understand the mechanisms of frameshift fidelity, it is important to investigate the interactions between DNA polymerases and misaligned primer−template structures. The Klenow fragment of DNA polymerase I is one of the most structurally well characterized members of the DNA polymerase family, making it a useful model system for structure−function studies of a DNA replication enzyme (reviewed in ref *5*). Proofreading by Klenow fragment is accomplished in a 3′−5′ exonuclease site that is separated from the polymerase site by 25−30 Å (*6*, *7*). We have previously reported a sensitive solution spectroscopic technique, based on the fluorescence anisotropy decay of a dansyl probe attached to DNA, that can be used to analyze the partitioning of a DNA substrate between the two active sites of the enzyme (*8*, *9*). This technique was used previously to study the effects of mismatches and unusual sequences on the partitioning of DNA substrates between the polymerase

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and 3′−5′ exonuclease sites (*9*, *10*). In addition, the same approach was used to evaluate the energetic contributions of individual amino acid residues to the binding of DNA substrates to the 3′−5′ exonuclease site of Klenow fragment (*11*).

The misaligned DNA intermediates in frameshift mutagenesis contain one or more extrahelical bases in either the primer or template strands, and these may provide features for recognition by the proofreading apparatus of DNA polymerases. To test this hypothesis, the time-resolved fluorescence anisotropy technique is used here to examine the interaction between Klenow fragment and bulged primer−templates containing a defined extrahelical base at various positions within the template strand. The results demonstrate that the presence of an extrahelical base near the 3′ terminus of the primer−template can significantly increase the occupancy of the 3′−5′ exonuclease site, suggesting that a DNA substrate will be transferred from the polymerase site to the exonuclease site following a misalignment event. Moreover, these effects are shown to depend on the nature of the extrahelical base and its position within the DNA substrate. Steady-state fluorescence measurements of analogous primer−templates containing the fluorescent base 2-aminopurine (AP) at the primer 3′ terminus are used to assess the effects of extrahelical bases on terminus unwinding and the kinetics of exonucleolytic cleavage of DNA. The results of this study provide insights into the physical mechanisms that enable DNA polymerases to recognize and correct frameshift errors during DNA replication.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus using standard β-cyanoethyl phosphoramidite chemistry and purified by reverse-phase HPLC. Oligonucleotide sequences are shown in Chart 1. Oligonucleotides labeled internally with a dansyl probe were prepared using a derivatizable uridine analogue as described previously (*12*). To prepare oligonucleotides with AP at the 3′ terminus, a DMT-protected 2-aminopurine was synthesized as described previously (*13*) and attached to a controlled-pore glass solid support (*14*). Unless otherwise noted, DNA duplexes were formed by mixing the primer oligonucleotide (17* or 17AP) with the appropriate template oligonucleotide in a small molar excess (10%), heating the resulting solution to 80 °C, and then allowing it to cool slowly to room temperature. The exonuclease-deficient D424A mutant of the Klenow fragment of DNA polymerase I was prepared as described previously (*15*). Wild-type Klenow fragment was purchased from Stratagene.

Formation of DNA−Protein Complexes. DNA−protein complexes were formed by adding D424A Klenow fragment to the dansyl-labeled primer−templates in a buffer of 50 mM Tris-HCl (pH 7.5) containing 3 mM $MgCl_2$. To establish the appropriate conditions for complex formation, DNA substrates (1−10 μM) were titrated with increasing amounts of Klenow fragment while the steady-state fluorescence intensity and anisotropy were being monitored after each addition. Protein was added until there was no further change in the steady-state fluorescence parameters. Titrations were carried out for bulged DNA substrates and the corresponding substrate with proper base pairing. In all cases, the titration profiles displayed a linear increase of intensity or anisotropy with an end point corresponding to equimolar amounts of DNA and enzyme. Accordingly, all DNA−protein complexes for time-resolved fluorescence measurements were prepared with 10 μM DNA (primer strand) and 13 μM enzyme.

Chart 1: Sequences of Primer−Templates[a]

17*/27-mer	5′-TCGCAGCCGX CCAAGGG-3′ 3′-AGCGTCGGCAGGTTCCCATATAGCCGA-5′
17*/28(1T)-mer	5′-TCGCAGCCGX CCAAGG G-3′ 3′-AGCGTCGGCAGGTTCC T CATATAGCCGA-5′
17*/28(1C)-mer	5′-TCGCAGCCGX CCAAGG G-3′ 3′-AGCGTCGGCAGGTTCC C CATATAGCCGA-5′
17*/28(1G)-mer	5′-TCGCAGCCGX CCAAGG G-3′ 3′-AGCGTCGGCAGGTTCC G CATATAGCCGA-5′
17*/28 (1A)-mer	5′-TCGCAGCCGX CCAAGG G-3′ 3′-AGCGTCGGCAGGTTCC A CATATAGCCGA-5′
17*/28(2A)-mer	5′-TCGCAGCCGX CCAAG GG-3′ 3′-AGCGTCGGCAGGTTC A CCATATAGCCGA-5′
17*/28(3A)-mer	5′-TCGCAGCCGX CCAA GGG-3′ 3′-AGCGTCGGCAGGTT A CCCATATAGCCGA-5′
17*/28(4A)-mer	5′-TCGCAGCCGX CCA AGGG-3′ 3′-AGCGTCGGCAGGT A TCCCATATAGCCGA-5′
17*/28(5A)-mer	5′-TCGCAGCCGX CC AAGGG-3′ 3′-AGCGTCGGCAGG A TTCCCATATAGCCGA-5′
17AP/27-mer	5′-TCGCAGCCGTCCAAGG(AP)-3′ 3′-AGCGTCGGCAGGTTCCTATATAGCCGA-5′
17AP/28 (1A)-mer	5′-TCGCAGCCGTCCAAGG (AP)-3′ 3′-AGCGTCGGCAGGTTCC A TATATAGCCGA-5′
17AP/28(4A)-mer	5′-TCGCAGCCGTCCA AGG(AP)-3′ 3′-AGCGTCGGCAGGT A TCCTATATAGCCGA-5′

[a] **X** denotes a deoxyuridine residue, modified at the C5 position with an amino propyl substituent conjugated to dansyl chloride. **AP** denotes 2-aminopurine.

Steady-State Fluorescence Spectroscopy. Steady-state emission spectra of primer−templates containing AP were obtained from 330 to 450 nm on an SLM 8100 spectrofluorimeter (Spectronic Instruments) by exciting at 310 nm. Solutions contained 10 μM 17AP primer and 30 μM template strands in 50 mM Tris-HCl (pH 7.5) and 3 mM $MgCl_2$ to ensure the absence of free 17AP strands.

Steady-State Exonuclease Cleavage Assays. The exonuclease activity of wild-type Klenow fragment was assayed on DNA substrates containing AP at the primer 3′ terminus (Chart 1). Solutions contained 1 μM 17AP primer and 1.1 μM template oligonucleotides in a buffer of 50 mM Tris-HCl (pH 7.5) containing 3 mM $MgCl_2$. The solutions were incubated at 20 °C in a quartz cuvette held in the sample chamber of the SLM8100 spectrofluorimeter. Reactions were

initiated by adding Klenow fragment directly to the cuvette, and the emission intensity of AP at 365 nm was subsequently monitored at 1 s intervals (excitation at 310 nm). Initial cleavage rates were calculated from the slope of the linear portion of the fluorescence increase versus time. Relative cleavage rates were calculated from the ratio of the slopes obtained for a bulged DNA substrate and the corresponding properly base-paired substrate.

Time-Resolved Fluorescence Spectroscopy. Fluorescence decay measurements were performed using the time-correlated single-photon counting setup described in detail elsewhere (*8*). Samples were measured in quartz cuvettes and excited at 318 nm using the vertically polarized output from a frequency-doubled synchronously mode-locked DCM dye laser (Coherent 702). The dansyl emission was monitored at 535 nm using a monochromator (JY H-10) and a microchannel plate photomultiplier (Hamamatsu R2809U-01). Time-resolved emission profiles were acquired using standard time-correlated single-photon counting electronics (Ortec and Tennelec). Decays were recorded in 512 channels of a multichannel analyzer (Ortec Norland 5510) using a sampling time of 88 ps per channel. For measurement of fluorescence anisotropy decay, a polarizer in the emission path was alternated between vertical and horizontal directions every 30 s, and the decays collected with each polarizer setting were accumulated in separate memory segments of the multichannel analyzer. Movement of the polarizer and data accumulation in the multichannel analyzer were under computer control. All measurements were performed at 20 °C. Time-resolved emission data were transferred to a Sun Sparc LX workstation for analysis.

Data Analysis. The time-dependent fluorescence anisotropy, $r(t)$, was calculated directly from polarized components of the time-resolved fluorescence:

$$r(t) = \frac{I_{||}(t) - I_{\perp}(t)}{I_{||}(t) + 2I_{\perp}(t)} \tag{1}$$

where $I_{||}(t)$ and $I_{\perp}(t)$ are intensity decays measured with the emission polarizer oriented parallel or perpendicular to the excitation polarization, respectively. The time-resolved anisotropy data were fitted to an expression allowing for two states of the dansyl probe, either solvent-exposed or buried, corresponding to binding of the primer 3′ terminus in the polymerase active site or the 3′−5′ exonuclease site of Klenow fragment, respectively (*8*, *9*, *11*). Accordingly, the observed time-dependent fluorescence anisotropy was represented by a sum of contributions from the exposed state, $r_e(t)$, and the buried state, $r_b(t)$:

$$r(t) = f_e(t)r_e(t) + f_b(t)r_b(t) \tag{2}$$

where the fractional contribution of exposed probes to the fluorescence anisotropy at time t, $f_e(t)$, is given by

$$f_e(t) = \frac{x_e\sum_{i=1}^{N_e}\alpha_{ie}\exp(-t/\tau_{ie})}{x_e\sum_{i=1}^{N_e}\alpha_{ie}\exp(-t/\tau_{ie}) + x_b\sum_{i=1}^{N_b}\alpha_{ib}\exp(-t/\tau_{ib})} \tag{3}$$

where τ_{ie}, α_{ie}, N_e, and x_e are the fluorescence lifetimes, decay amplitudes, number of decay components, and ground-state mole fraction of the exposed dansyl probes, respectively. The corresponding parameters for the buried probes are denoted with a subscript b. An analagous expression was used to represent the fractional contribution of the buried probes, $f_b(t)$.

The intrinsic anisotropy decay function for each probe population was represented as a sum of contributions from local rotation of the dansyl probe and overall rotation of the DNA−protein complex, as shown in eq 4 for the exposed probes:

$$r_e(t) = \beta_{1e}\exp(-t/\phi_{1e}) + \beta_{2e}\exp(-t/\phi_{2e}) \tag{4}$$

where β_{1e} and ϕ_{1e} are the anisotropy amplitude and decay time for local rotation of the exposed probes, respectively, and β_{2e} and ϕ_{2e} are the corresponding quantities for overall tumbling. A similar expression was used to represent the time-dependent anisotropy of the buried dansyl probes. The formalism presented here does not allow for convolution effects, but these were considered to be negligible in light of the narrow width of the instrument response function (45 ps fwhm) relative to the time scale of the motions involved (≥1 ns).

The fractions of exposed and buried dansyl probes, corresponding to the fractions of DNA primer−templates bound at the polymerase or 3′−5′ exonuclease sites, respectively, were obtained by globally fitting multiple anisotropy data sets to the two-state model. The fluorescence lifetimes and decay amplitudes for each population were fixed at values recovered from a global analysis of Klenow fragment−17/27-mer complexes reported previously (*9*). The rotational correlation times were also fixed at the previously determined values, while the anisotropy amplitudes were globally linked and optimized across all data sets. The fraction of buried probes (x_b) was optimized for each data set during the global analysis. The anisotropy data were assigned appropriate weighting factors according to the values of $I_{||}(t)$ and $I_{\perp}(t)$ at each time point (*16*). The quality of the fits was judged by the reduced χ^2 value for each data set. The equilibrium constant for partitioning of DNA from the polymerase site to the 3′−5′ exonuclease site, K_{pe}, was calculated from the fitted fractions of buried probes according to eq 5:

$$K_{pe} = \frac{x_b}{1 - x_b} \tag{5}$$

RESULTS

Partitioning of Bulged Primer−Templates between Polymerase and 3′−5′ Exonuclease Sites. A series of dansyl-labeled 17*/28-mer primer−template oligonucleotides were designed to examine the effects of unpaired extrahelical bases upon the binding of DNA substrates to Klenow fragment (Chart 1). Bulged DNA substrates were formed by introducing an extra unpaired base into the template strand of an otherwise perfectly base-paired 17*/27-mer primer−template. Such structures would give rise to deletion mutations during DNA synthesis. The identity of the extra base and its position within the template oligonucleotide were both varied. The primer strand (17*) was labeled with a dansyl probe attached

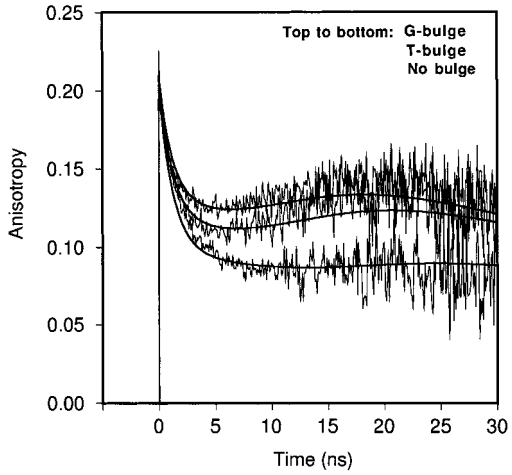

FIGURE 1: Effects of different extrahelical bases on the interaction between Klenow fragment and bulged DNA substrates. Shown are fluorescence anisotropy decay profiles of dansyl-labeled 17*/28-mer primer−templates bound to D424A Klenow fragment. The primer−templates contain different extrahelical template bases at the first position from the primer 3′ terminus, as indicated. Data for the corresponding primer−template with proper base pairing (17*/27-mer) are also shown for comparison. See Chart 1 for complete DNA sequences. The fitted curves (solid lines) are from a global fit to a two-state model. See the text for details.

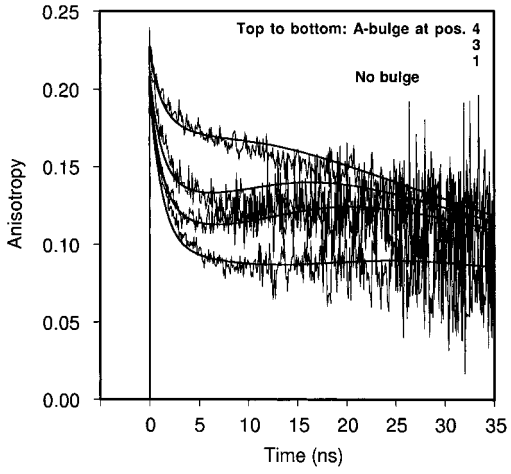

FIGURE 2: Recognition of extrahelical bases at different positions within the DNA substrate. Fluorescence anisotropy decays of dansyl-labeled primer−templates bound to D424A Klenow fragment are shown. The primer−templates contain an extrahelical adenine base at increasing distances from the primer 3′ terminus, as indicated. Data for the properly base-paired substrate are also shown for comparison. The solid lines are from a global fit to a two-state model, as discussed in the text.

to a uridine residue located seven bases from the 3′ terminus. This position is particularly sensitive to the mode of binding to the polymerase (*8*, *9*). Bulges were introduced into the template strand, rather than into the primer strand, to minimize any direct effect of the unpaired bases on the dansyl probe. Indeed, all the dansyl-labeled primer−templates exhibited similar fluorescence intensity and anisotropy decays when free in solution, with an average fluorescence lifetime of 4 ns and a rotational correlation time of 10 ns (not shown).

The time-resolved fluorescence anisotropy decay of a dansyl probe can be used to monitor simultaneously the binding of labeled DNA substrates to the polymerase site and 3′−5′ exonuclease site of Klenow fragment (*8*, *9*). The precise shape of the decay profile is strongly dependent upon the relative fractions of the two bound forms. Accordingly, fluorescence anisotropy decays were recorded for the bulged 17*/28-mer substrates bound to D424A Klenow fragment and compared with the corresponding decay for the properly base-paired 17*/27-mer substrate. The exonuclease-deficient D424A mutant Klenow fragment was used to ensure that the DNA substrates were not degraded during the course of the fluorescence decay measurements. The lack of exonuclease activity in the D424A mutant is due to the loss of a catalytically essential magnesium ion (*17*), but the enzyme appears to have little or no defect in its ability to bind DNA substrates at the 3′−5′ exonuclease site (*11*).

Figure 1 illustrates the effects of extrahelical purine (guanine) and pyrimidine (thymine) bases at the first position from the primer 3′ terminus. It is evident that the anisotropy decay profiles for the bulged substrates are quite different from that for the properly base-paired substrate, reflecting the influence of the extrahelical bases on binding to the polymerase. Moreover, it is apparent that the anisotropy decays are sensitive to the nature of the extrahelical base in these bulged DNA substrates.

Figure 2 illustrates the effects of an embedded extrahelical adenine base at different positions within the template strand of a DNA substrate bound to Klenow fragment. The anisotropy decays are markedly different from that observed for the properly base-paired primer−template, indicating that the polymerase can recognize extrahelical bases located upstream from the primer 3′ terminus. It is also apparent that the extrahelical adenine base has a different effect at each position within the DNA substrate.

The anisotropy decay profiles for the entire set of DNA−protein complexes (including all the dansyl-labeled primer−templates shown in Chart 1) were globally analyzed using a two-state model of exposed and buried dansyl probes (eqs 2−4). For these fits, the intrinsic donor lifetimes and decay amplitudes were fixed at the values established in a previous analysis of 17*/27-mers bound to Klenow fragment (*9*). The following values were used: $\tau_{1e} = 1.01$ ns, $\tau_{2e} = 4.13$ ns, $\tau_{3e} = 11.9$ ns, $\alpha_{1e} = 0.30$, $\alpha_{2e} = 0.67$, $\alpha_{3e} = 0.03$, $\tau_{1b} = 3.06$ ns, $\tau_{2b} = 18.4$ ns, $\alpha_{1b} = 0.70$, and $\alpha_{2b} = 0.30$. The anisotropy decay times were also fixed at previously determined values ($\phi_{1e} = \phi_{1b} = 1.24$ ns, $\phi_{2e} = \phi_{2b} = 57.2$ ns), whereas the corresponding amplitudes were globally optimized across all decays. The resulting values ($\beta_{1e} = 0.115$, $\beta_{2e} = 0.093$, $\beta_{1b} = 0.023$, and $\beta_{2b} = 0.222$) are in close accord with previous results (*9*). During the global fitting, the fractions of buried dansyl probes were optimized for each decay. Typical fitted curves are shown by the solid lines in Figures 1 and 2. Satisfactory fits were obtained in almost all cases (reduced χ^2 values are given in Table 1), indicating that the anisotropy decays for the bulged substrates can be represented in terms of two states of the dansyl probe, with the same fluorescence lifetime and rotational properties observed previously for the binding of regular duplexes to Klenow fragment. Thus, the exposed and buried dansyl probes can be associated with DNA substrates bound to the polymerase site or the 3′−5′ exonuclease site, respectively, as established previously (*9*, *11*).

The partitioning constant, K_{pe}, describing the distribution of DNA between the polymerase and 3′−5′ exonuclease sites was calculated from the fraction of buried dansyl probes recovered from the global analysis (eq 5). The partitioning

Table 1: Partitioning of DNA between Polymerase and 3′−5′ Exonuclease Sites[a]

Substrate	Terminal sequence[b]	χ^2[c]	K_{pe}[d]
17*/27-mer	X CCAAGGG-3′ AGGTTCCC	1.6	0.034
17*/28 (1T)-mer	X CCAAGG G-3′ AGGTTCC T C	1.4	0.173
17*/28 (1C)-mer	X CCAAGG G-3′ AGGTTCC C C	1.4	0.121
17*/28 (1G)-mer	X CCAAGG G-3′ AGGTTCC G C	2.4	0.287
17*/28 (1A)-mer	X CCAAGG G-3′ AGGTTCC A C	1.2	0.179
17*/28 (2A)-mer	X CCAAG GG-3′ AGGTTC A CC	1.3	0.105
17*/28 (3A)-mer	X CCAA GGG-3′ AGGTT A CCC	2.1	0.398
17*/28 (4A)-mer	X CCA AGGG-3′ AGGT A TCCC	1.6	1.60
17*/28(5A)-mer	X CC AAGGG-3′ AGG A TTCCC	1.9	0.133

[a] Anisotropy decays were globally fitted to eq 2, using the fluorescence lifetime and rotational decay parameters determined previously (*9*). [b] The bases near the primer 3′ terminus are shown. **X** denotes the position of a dansyl-labeled uridine residue. See Chart 1 for complete DNA sequences. [c] Local reduced χ^2 value from global fitting of the anisotropy decay to eq 2. [d] Equilibrium constant for partitioning of DNA between polymerase and 3′−5′ exonuclease sites. Errors in K_{pe} are approximately ±15%.

constants for the bulged 17*/28-mers and the properly base-paired 17*/27-mer are presented in Table 1. In general, the K_{pe} values for the bulged substrates are larger than those for the correct substrate, showing that the extrahelical bases promote transfer of DNA to the 3′−5′ exonuclease site. The presence of an extrahelical template base at the first position from the primer 3′ terminus increases the partitioning constant by a factor of 3−8 relative to that of the properly base-paired substrate (Table 1). These effects are comparable to those observed previously for a single mismatched base pair at the primer 3′ terminus (*9*). Moreover, the ability of different extrahelical bases to promote partitioning of DNA into the 3′−5′ exonuclease site can be ranked in the following order: G > A ≈ T > C (Table 1). Note that in the case of the bulged substrate with an extra cytosine in the template, the extrahelical base in principle can also be located at the second and third positions upstream from the primer 3′ terminus. Thus, the results for this substrate may also reflect the variable position of the extrahelical base.

The results in Table 1 indicate that an extrahelical base is also recognized up to five bases from the primer 3′ terminus. Moreover, the precise effect of an embedded extrahelical base on partitioning is strongly dependent upon its position within the DNA. The largest effects are observed for the extrahelical adenine base at the third or fourth positions from the primer 3′ terminus, which increase the partitioning constant by 12- and 47-fold, respectively, relative to that of the properly base-paired substrate.

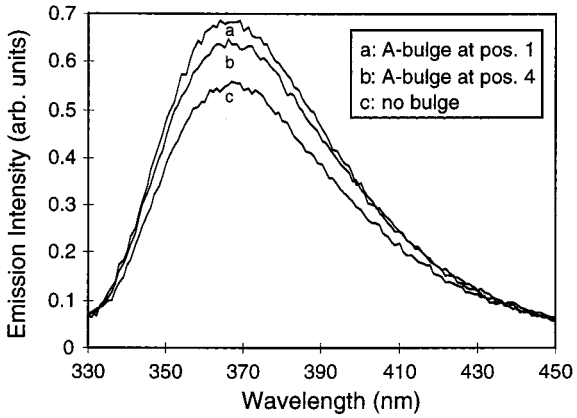

FIGURE 3: Fluorescence emission spectra of 2-aminopurine (AP). Spectra are shown for primer−templates containing AP at the primer 3′ terminus. (a) 17AP/28(1A)-mer, with an extrahelical adenine base at the first position from the primer 3′ terminus. (b) 17AP/28(4A)-mer, with an extrahelical adenine base at the fourth position from the terminus. (c) 17AP/27-mer, properly base-paired primer−template. See Chart 1 for complete base sequences. Spectra were recorded at 20 °C by exciting at 310 nm. Solutions contain 10 μM 17AP and 30 μM template oligonucleotides. Other solution conditions are given in Materials and Methods.

Effects of Extrahelical Bases on Unwinding of the Primer 3′ Terminus. The introduction of extrahelical bases into the DNA substrates may promote unwinding and fraying of the primer 3′ terminus. To examine this possibility, steady-state fluorescence measurements were performed using primer−templates containing AP at the primer 3′ terminus (Chart 1). The fluorescence of AP is strongly quenched upon incorporation of the base into duplex DNA. Accordingly, AP can be used to probe the extent of base pairing at a specific site in duplex DNA (*18*−*20*). In particular, structural changes that disrupt base pairing are manifested as an increase in the emission of AP. Figure 3 illustrates the steady-state fluorescence emission properties of AP in both properly base-paired and bulged primer−templates. These DNA substrates are analagous to those used for the partitioning measurements except that the dansyl probe is absent (the modified uridine residue is replaced by thymidine) and the guanine base at the primer 3′ terminus is replaced by AP (the corresponding template base is changed to thymine which is complementary to AP; see Chart 1). The excitation maximum for AP is at 310 nm (not shown), and the emission peak is at 365 nm for each primer−template. The emission intensity is significantly higher in the primer−templates containing an extrahelical adenine base than in the properly base-paired primer−template, indicating that AP is less quenched due to base−base interactions (*19*, *20*). These results reveal that the 3′ terminal AP base is less well paired in the bulged primer−templates, although they do not indicate the actual degree of terminus unwinding. However, on the basis of the relative emission intensities of AP, it is evident that the base pairing is disrupted to a lesser extent when the extrahelical base is at the fourth rather than first position from the primer 3′ terminus (Figure 3). Taken together, these results indicate that the introduction of extrahelical bases into the DNA primer−templates increases the degree of terminus unwinding and that the effect is greatest when the extrahelical base is close to the primer terminus.

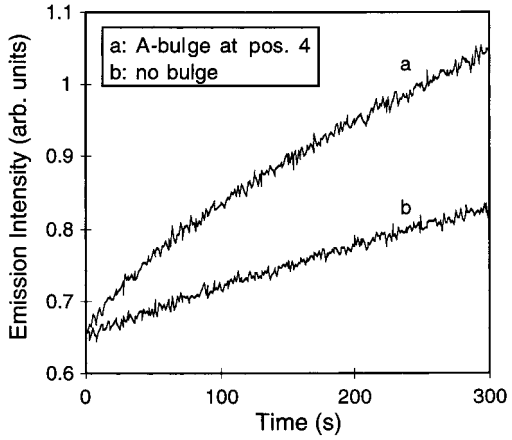

FIGURE 4: Time courses for excision of AP from primer−templates with and without extrahelical bases. (a) 17AP/28(4A)-mer, with an extrahelical adenine base at the fourth position from the primer 3′ terminus. (b) 17AP/27-mer, properly base-paired primer−template. The fluorescence emission intensity of AP at 365 nm is shown for reactions initiated by addition of wild-type Klenow fragment to a solution of the specified primer−template at 20 °C. Final concentrations are 1.0 μM DNA (primer strand) and 0.29 μM enzyme. Other solution conditions are given in Materials and Methods.

Steady-State Exonuclease Cleavage Activity. The increased partitioning of DNA substrates into the 3′−5′ exonuclease site resulting from the introduction of extrahelical bases may also be reflected in an enhanced rate of DNA cleavage activity. To test this possibility, the steady-state kinetics of removal of AP from the primer 3′ terminus of various DNA substrates were measured by monitoring the increase in fluorescence emission as AP was excised (*21*). Figure 4 depicts the fluorescence time courses obtained after addition of wild-type Klenow fragment to a solution of 17AP/28-(4A)-mer (curve a) or 17AP/27-mer (curve b). The former substrate contains an extrahelical adenine base at the fourth position from the primer 3′ terminus, which is observed to have the greatest effect on partitioning between polymerase and 3′−5′ exonuclease sites (Table 1), while the latter substrate is properly base-paired. Reaction mixtures contained approximately 0.29 μM Klenow fragment and 1.0 μM DNA (primer strand) and are the result of multiple turnovers of substrate to product. It is evident that the steady-state rate of cleavage of AP is substantially faster in the bulged primer−template. Apparent cleavage rates were estimated from the slopes of the initial linear portion of the fluorescence time traces. On the basis of the ratio of these initial rates, it is found that AP is excised from the bulged substrate 4.2-fold faster than from the correctly base-paired substrate.

DISCUSSION

This study was undertaken to test the hypothesis that extrahelical bases within a DNA substrate provide recognition features for the proofreading machinery of DNA polymerases. Such recognition would form the basis for exonucleolytic proofreading of the misaligned premutational DNA intermediates that arise by primer−template slippage in repetitive DNA sequences. Indeed, the results presented here demonstrate that the presence of an extrahelical base within a DNA primer−template does have a pronounced effect on how the DNA partitions between the polymerase and 3′−5′ exonuclease sites of a DNA polymerase. The presence of a single extrahelical template base at the first position from the primer 3′ terminus (corresponding to a deletion mutation) caused a 3−8-fold increase in the level of partitioning of DNA into the 3′−5′ exonuclease site, depending on the nature of the extrahelical base. These effects are comparable to those previously observed for single mismatches at the primer 3′ terminus (*9*).

In principle, this increase in partitioning of DNA from the polymerase site to the 3′−5′ exonuclease site could reflect two different situations. First, an extrahelical base might increase the intrinsic affinity of DNA for the exonuclease site without changing the affinity for the polymerase site. Alternatively, the extrahelical base might interfere with DNA binding at the polymerase site without affecting the affinity of the DNA for the exonuclease site. The intrinsic affinity of the exonuclease site for a duplex DNA substrate is correlated with the thermodynamics of terminus unwinding, because the primer 3′ terminus binds to the exonuclease site as a single-stranded end (*22*−*24*). Consistent with this, fluorescence measurements employing the fluorescent base 2-aminopurine indicate that an extrahelical template base adjacent to the primer 3′ terminus does disrupt base pairing and increase the degree of terminus unwinding. This observation suggests that bulged DNA substrates with extrahelical bases close to the primer 3′ terminus partition in favor of the exonuclease site because of the annealing state of the substrate. In addition, unfavorable steric interactions at the polymerase site might make an additional contribution to the increased level of partitioning observed for these bulged DNA substrates relative to that of properly base-paired DNA. In fact, extrahelical purine bases were found to be generally more effective in promoting partitioning of DNA into the 3′−5′ exonuclease site than extrahelical pyrimidines (Table 1), which may reflect greater steric interference within the polymerase domain owing to the larger size of the purines.

We also examined DNA substrates in which the extrahelical template base was more distant from the primer 3′ terminus, to test whether the polymerase could recognize embedded extrahelical bases within a region of correct base pairing. Such structures potentially can be formed by primer−template slippage in a homopolymeric run of two or more bases. In fact, extrahelical bases located up to five bases from the primer 3′ terminus strongly promote partitioning of the DNA substrates into the 3′−5′ exonuclease site (Table 1). Interestingly, extrahelical bases at the third and fourth positions from the primer 3′ terminus increase the level of partitioning to the exonuclease site to a much greater extent than equivalent extrahelical bases immediately adjacent to the terminus. The large effects caused by bulges at these positions cannot be attributed to an increased degree of fraying of the primer terminus because the AP fluorescence data indicate that the degree of terminus unwinding is actually smaller than that for an extrahelical base adjacent to the primer terminus (Figure 3). This suggests that the embedded extrahelical bases exert their effect on partitioning primarily by interfering with the binding of the DNA substrates to the polymerase domain of the enzyme. Such a mechanism would result in weaker overall binding of the bulged DNAs to the enzyme. Unfortunately, it is not possible to measure the dissociation constants for binding of any of the DNA substrates to Klenow fragment because of the

limited sensitivity of the dansyl probe, which does not permit the use of DNA concentrations below 1 μM. The dissociation constant for the binding of duplex DNA to Klenow fragment is in the low nanomolar range (*25*), necessitating the use of much lower DNA concentrations for binding measurements. Accordingly, we cannot directly address the question of whether the embedded extrahelical bases actually interfere with the binding of DNA substrates to the polymerase domain. However, this is the most likely explanation given that the observed effects cannot be accounted for in terms of an increased level of fraying of the primer terminus. Moreover, the possibility that extrahelical bases located up to five bases from the primer terminus can interfere with binding of DNA to the polymerase site is consistent with previous chemical footprinting and fluorescence spectroscopic studies indicating that the polymerase domain of Klenow fragment makes contact with 5−8 bp of duplex DNA (*8*, *26*, *27*).

The findings reported here complement previous studies of the effects of mismatches and unusual DNA sequences on the interactions between Klenow fragment and duplex DNA (*9*, *10*). Using the time-resolved fluorescence anisotropy technique, it was observed that mismatches at the primer 3′ terminus promoted transfer of DNA to the 3′−5′ exonuclease site, consistent with a terminus that is more easily melted (*9*). Interestingly, internal mismatches located up to 4 bp from the primer terminus were found to be more effective in promoting transfer of DNA to the 3′−5′ exonuclease site than equivalent mismatches at the primer terminus itself (*9*), suggesting that the mismatches interfered with the binding of duplex DNA to the polymerase domain of the enzyme. These results are analogous to the present data about the effect of embedded extrahelical bases, although the effects observed here for extrahelical bases at the third and fourth positions from the primer 3′ terminus are larger than those observed for internal mismatches at the same positions (*9*). A recent study has shown that the presence of an A-tract sequence element at the primer terminus can also favor binding of a duplex DNA substrate to the 3′−5′ exonuclease site, despite the absence of mismatches within the DNA (*10*). This effect was ascribed to an unusual structure adopted by the A-tract which was disruptive to binding of the duplex DNA to the polymerase site. The results of these studies, together with the present data on bulged DNA substrates, suggest that the local structure of the primer−template duplex can exert a significant influence on the partitioning of DNA between the polymerase and 3′−5′ exonuclease sites.

A potential problem in the interpretation of the experiments described here is that the presence of an extrahelical base might alter the mode of binding of a DNA substrate to the polymerase site or to the 3′−5′ exonuclease site, which could alter the resulting probe environments. If so, it would not be possible to fit the anisotropy decays using the fluorescence lifetime and rotational parameters previously established for the exposed and buried dansyl probes. However, the anisotropy decays obtained for primer−templates containing an extrahelical template base at the first or second positions from the primer 3′ terminus yielded excellent fits, indicating that the two probe environments are not significantly different from those observed for binding of regular duplexes to Klenow fragment. This is expected for the exonuclease mode of binding (buried probes) because of the localized melting and strand separation that occur around the primer 3′ terminus when DNA primer−templates bind to the 3′−5′ exonuclease site. Previous studies indicate that 3 or 4 bp of duplex DNA at the 3′ terminus are melted out during binding to the 3′−5′ exonuclease site (*22*−*24*). Thus, a bulge within this region of the primer−template should not alter the mode of binding at the exonuclease site. Moreover, on the basis of the good fits obtained, it appears that an extrahelical base at the first or second positions from the primer 3′ terminus does not appreciably alter the mode of binding of the primer−templates to the polymerase site either (exposed probes).

However, in the case of an extrahelical template base located at the third, fourth, and fifth positions from the primer 3′ terminus, the fits to the original two-state model are not quite as good (Table 1). This probably reflects a change in the polymerase mode of binding, rather than in the exonuclease mode, because the extrahelical bases at these positions are still expected to be melted out when the primer−templates bind to the 3′−5′ exonuclease site. Nonetheless, the perturbations induced by the extrahelical base may be relatively small, given that the fits to the two-state model still provide a reasonable representation of the anisotropy decays observed for the DNA substrates with bulges at the internal positions (Figure 2 and Table 1). Subtle changes in the polymerase mode of binding for these particular substrates are probably a reflection of the difficulty of accommodating the extrahelical bases within the polymerase domain of the enzyme, which is also manifested in the corresponding K_{pe} values (Table 1).

The most important observation from this study is that extrahelical bases within a DNA primer−template cause the DNA to partition in favor of the 3′−5′ exonuclease site. Thus, misalignment of primer and template strands and the subsequent formation of an extrahelical base will promote transfer of the DNA into the exonuclease domain of the polymerase. The exonuclease domain could assist in the correction of these premutational intermediates by exonucleolytic cleavage of the DNA. Consistent with this, we observed an increased rate in DNA cleavage activity for a bulged DNA substrate (containing an extrahelical adenine at the fourth position from the primer terminus) relative to a properly base-paired substrate (Figure 4). The presence of the extrahelical base causes a 4.2-fold enhancement of the steady-state rate of cleavage of the 3′ terminal AP base, which is correlated with the higher level of partitioning to the exonuclease site relative to that of properly base-paired DNA (Table 1), although the change in the cleavage rate is quantitatively smaller than the change in the partitioning constant. The rate of exonuclease cleavage observed under multiple-turnover conditions reflects a number of kinetic processes, including translocation of DNA from the polymerase site to the 3′−5′ exonuclease site, local melting and unwinding of the primer terminus, hydrolysis of the scissile phosphodiester bond, and release of the mononucleotide product into solution. Accordingly, it is not possible to predict a priori the relationship between the equilibrium constant for partitioning of a DNA substrate between the polymerase and 3′−5′ exonuclease sites and the corresponding steady-state rate of exonucleolytic cleavage. In addition, the primer−templates used for the partitioning and cleavage measure-

ments are not identical (Chart 1), which also complicates a direct comparison of the partitioning constant and the exonucleolytic cleavage rate. Nonetheless, these results establish a link between the exonuclease cleavage activity and the higher level of partitioning of bulged primer−templates relative to those of properly base-paired DNA. Thus, preferential partitioning of misaligned DNA primer−templates into the 3′−5′ exonuclease domain represents an important step in the proofreading mechanism and frameshift fidelity of DNA polymerases.

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