Pre-Steady-State Kinetic Analysis of Sequence-Dependent Nucleotide Excision by the 3′-Exonuclease Activity of Bacteriophage T4 DNA Polymerase†

Linda B. Bloom,‡ Michael R. Otto,§ Ramon Eritja,∥ Linda J. Reha-Krantz,⊥ Myron F. Goodman,‡ and Joseph M. Beechem*,§

Department of Biological Sciences, Molecular Biology Section, University of Southern California, Los Angeles, California 90089-1340, Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232, Department of Molecular Genetics, Center for Research and Development, Jordi Girona 18-26, 08034 Barcelona, Spain, and Department of Genetics, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

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ABSTRACT: The effects of local DNA sequence on the proofreading efficiency of wild-type T4 DNA polymerase were examined by measuring the kinetics of removal of the fluorescent nucleotide analog 2-aminopurine deoxynucleoside monophosphate (dAPMP) from primer/templates of defined sequences. The effects of (1) interactions with the 5′-neighboring bases, (2) base pair stability, and (3) G·C content of the surrounding sequences on the pre-steady-state kinetics of dAPMP excision were measured. Rates of excision dAPMP from a primer 3′-terminus located opposite a template T (AP·T base pair) increased, over a 3-fold range, with the 5′-neighbor to AP in the order C < G < T < A. Rates of removal of dAPMP from AP·X base pairs located in the same surrounding sequence increased as AP·T < AP·A < AP·C < AP·G, which correlates with the decrease in the stabilities of these base pairs predicted by T_m measurements. A key finding was that AP was excised at a slower rate when mispaired opposite C located next to four G·C base pairs than when correctly paired opposite T next to four A·T base pairs, suggesting that exonuclease mismatch removal specificities may be enhanced to a much greater extent by instabilities of local primer termini than by specific recognition of incorrect base pairs. In polymerase-initiated reactions, biphasic reaction kinetics were observed for the excision of AP within most but not all sequence contexts. Rates of the rapid phases (30–40 s^{-1}) were relatively insensitive to sequence context. Rapid-phase rates reflect the rate constants for exonucleolytic excision of dAPMP from melted primer termini for *both* correct and incorrect base pairs and were roughly comparable to rates of removal of dAPMP from single-stranded DNA (65–80 s^{-1}). Rates of the slow phases (3–13 s^{-1}) were dependent on sequence context; the slow phase may reflect the rate of switching from the polymerase to the exonuclease active site, or perhaps the conversion of a primer/template terminus from an annealed to a melted state in the exonuclease active site. These data, using wild-type T4 DNA polymerase and two exonuclease-deficient T4 polymerases, support a model in which exonuclease excision occurs on melted primer 3′-termini for both mismatched and correctly matched primer termini, and where specificity favoring removal of terminally mismatched base pairs is determined by the much larger fraction of melted-out primer 3′-termini for mispairs compared to that for correct pairs.

DNA polymerases are responsible for accurate replication of DNA, and many polymerases have an associated 3′ → 5′ proofreading exonuclease activity either as part of the same polypeptide or as a separate subunit. The presence of proofreading exonuclease activity enables a polymerase to correct errors made during DNA synthesis (Brutlag & Kornberg, 1972; Muzyczka *et al.*, 1972). After a nucleotide is inserted, a polymerase can do one of three things: (1) go forward and extend the primer, possibly extending a mismatch, (2) remove the newly inserted nucleotide, or (3) dissociate from the DNA. The balance between further extension and exonucleolytic proofreading makes an important contribution to fidelity of DNA synthesis (Muzyczka *et al.*, 1972; Clayton *et al.*, 1979; Fersht *et al.*, 1982). A polymerase with a greater propensity to go forward and extend mispairs easily in competition with proofreading or dissociation will be error prone (Muzyczka *et al.*, 1972; Bessman *et al.*, 1974; Clayton *et al.*, 1979). On the other hand, a polymerase that removes nucleotides too frequently will waste nucleotides, which will be costly to the organism (Clayton *et al.*, 1979; Fersht *et al.*, 1982). A polymerase that dissociates too readily will lead to inefficient replication.

In addition to the intrinsic polymerase and exonuclease activities of an enzyme, local DNA sequence and structure can contribute to the fidelity of DNA synthesis (Pless & Bessman, 1983; Petruska & Goodman, 1985; Mendelman *et al.*, 1989; Kunkel, 1990; Joyce *et al.*, 1992; Bloom *et al.*, 1993). Local DNA sequence may affect the relative stability of a duplex at the primer 3′-terminus, which may in turn affect the efficiency of exonucleolytic proofreading. Both the X-ray crystal structure (Freemont *et al.*, 1988; Beese *et al.*, 1993) and cross-linking studies (Cowart *et al.*, 1989) for Klenow fragment (the large proteolytic fragment of *Escherichia coli* DNA polymerase I) suggest that four or five nucleotide units at a primer terminus must dissociate from the template in

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‡ University of Southern California.
§ Vanderbilt University.
∥ Center for Research and Development.
⊥ University of Alberta.

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order for the primer 3′-end to translocate from the polymerase to the exonuclease active site. The exonucleases of Klenow fragment and T4 polymerase are not active on cross-linked primer/templates in which the 3′-terminal four and two nucleotide units, respectively, are incapable of dissociating from the template (Cowart *et al.*, 1989). These data suggest that a primer terminus must be capable of local dissociation from the template in order to be a substrate for a 3′ → 5′ exonuclease. If this is the case, then local DNA sequences that help to stabilize the duplex region at a primer terminus would be expected to decrease the efficiency of an exonuclease (Bessman & Reha-Krantz, 1977). Changes in the local DNA sequence at a primer terminus that destabilize the duplex would be expected to increase the efficiency of exonucleolytic proofreading. In this paper, we explore effects that local DNA sequence variations can have on the proofreading efficiency of T4 DNA polymerase. T4 DNA polymerase is a single polypeptide containing both 5′ → 3′ polymerase and 3′ → 5′ exonuclease activities. As a key member of a multi-subunit replication complex, T4 DNA polymerase is responsible for the replication of the T4 genome; for recent reviews, see Nossal, (1992) and Young *et al.* (1992).

2-Aminopurine (AP),[1] an isomer of adenine with an amino group in the 2-position rather than the 6-position, was used as a probe to evaluate the influences of DNA sequence on proofreading efficiency. The position of the amino group affects the spectral properties of the base such that the absorption maximum of AP is shifted to a longer wavelength (304 nm) and AP is much more fluorescent than A (Ward *et al.*, 1969). AP is still capable of forming a Watson–Crick type base pair with thymine and can be accommodated within the structure of normal B-type DNA (Sowers *et al.*, 1986; Nordlund *et al.*, 1989). Polymerases are able to utilize dAPTP as a substrate for insertion, and 3′ → 5′exonucleases are able to excise dAPMP (Muzyczka *et al.*, 1972; Bessman *et al.*, 1974; Clayton *et al.*, 1979). In *in vitro* experiments, AP deoxynucleotide is inserted with a frequency of about 10–15% when competing with A for insertion opposite T (Bessman *et al.*, 1974; Clayton *et al.*, 1979; Pless & Bessman, 1983). AP is also capable of forming a less stable base pair with C, either as a protonated Watson–Crick structure or as a wobble structure, depending on pH (Sowers *et al.*, 1986, 1989). In competition with G, AP is misincorporated opposite C about 1% of the time (Mhaskar & Goodman, 1984), and when AP is present on a template, C is misincorporated at a frequency of about 5% in competition with T (Watanabe & Goodman, 1981). The ability of AP to compete with A for insertion opposite T and to form a relatively stable mispair with C causes it to be mutagenic, resulting in both A·T → G·C and G·C → A·T transitions *in vivo* (Freese, 1959; Hopkins & Goodman, 1979; Ronen, 1979). Although AP has been shown to form a wobble-type base pair with A in double-stranded DNA, polymerases are not easily able to insert dAPMP opposite A, which may reflect geometric constraints within the polymerase active site (Mhaskar & Goodman, 1984).

The effects of three different aspects of local DNA sequence context on proofreading efficiency by T4 pol were examined using AP as a probe. The efficiency of excision of AP was measured for (1) primer/templates (P/Ts) containing a "correct" Watson–Crick type AP·T base pair in comparison with AP·A, AP·C, and AP·G mispairs, (2) P/Ts with each of the four possible nucleotide bases 5′ to AP, and (3) P/Ts which differ in the number of G·C and A·T base pairs in the region of DNA immediately 5′ to AP. Primer/templates contained AP at the primer 3′-terminus. P/Ts were constructed so that within a given series only a single aspect of the DNA sequence was changed so that the effects of this change could be evaluated in the absence of other sequence effects (Table 1).

Table 1: Oligonucleotide Sequences for Primer/Templates Containing 2-Aminopurine at the Primer 3′-Terminus

(1) Primer/Templates with Different P·X[a] Base Pairs and 5′-Nearest Neighbors

17mer primer:

5′TCC CAG TCA CGA CGT **NP**

30mer template:

3′AGG GTC AGT GCT GCA **MXA** GTA CGA GCT ACT

primer	N	template	M	X
pGP	G	tCT	C	T
pTP	T	tAT	A	T
pAP	A	tTT	T	T
pCP	C	tGT	G	T
		tGA	G	A
		tGC	G	C
		tGG	G	G

(2) Primer/Templates with Different G·C versus A·T Content

17mer primer:

5′TCG CAG GAC TAC **NNN NP**

30mer template:

3′AGC GTC CTG ATG **MMM MXA** ACT AGA GCT ACT

primer	NNNN	template	MMMM	X
pGC	CGGC	tGC_T	GCCG	T
		tGC_C	GCCG	C
pAT	TAAT	tAT_T	ATTA	T
		tAT_C	ATTA	C

[a] The symbol P is used to represent 2-aminopurine when the analog is incorporated at a primer 3′-terminus.

EXPERIMENTAL PROCEDURES

Materials

Wild-type T4 DNA polymerase and exonuclease-deficient mutants D112A/E114A and D219A were prepared and purified as described previously (Reha-Krantz & Nonay, 1993; Reha-Krantz *et al.*, 1993). T4 pol and mutant concentrations were determined by the absorbance at 280 nm [1.492×10^5 M^{-1} cm^{-1} (Reha-Krantz *et al.*, 1991)]. Each preparation of T4 pol was titrated to determine the concentration of T4 pol necessary to completely bind DNA primer/templates. Titrations were done by two methods. (1) The increase in fluorescence intensity was monitored as T4 pol was added to AP-labeled DNA in the presence of EDTA. The intensity increased as the concentration of the T4 pol·DNA complex increased and reached a maximum when DNA was saturated with T4 pol. (2) Excision of dAPMP was measured as a function of T4 pol concentration. The maximal rate of dAPMP excision was presumed to indicate the T4 pol concentration at which all of the DNA was bound to the enzyme. DNA (200 nM) was saturated with T4 pol when the concentration of T4 pol was 2 times as great as the concentration of DNA.

Oligonucleotides were prepared using standard β-cyanoethylphosphoramidite reagents from Applied Biosystems. AP was incorporated at the 3′-end using an AP-derivatized CPG (Connolly, 1991). Primer/templates were annealed in buffer

[1] Abbreviations: AP, 2-aminopurine; P, 2-aminopurine when incorporated within a single- or double-stranded DNA polymer; T4 pol, wild-type T4 DNA polymerase; P/T, primer/template; ds, double-stranded; ss, single-stranded; NBS, *N*-bromosuccinimide; PMT, photomultiplier tube.

containing 25 mM HEPES, pH 7.5, and 50 mM NaCl. The reaction mixtures were first heated to 90 °C and then slowly cooled to room temperature. Template concentrations were 20% greater than primer concentrations. To determine an appropriate ratio of template to primer for annealing reactions, insertion of dAMP opposite T was measured at 37 °C using the exonuclease-deficient mutant D112A/E114A for primer/templates identical to those with different 5′-neighbors (Table 1) except that the primer was missing two nucleotides at the 3′end. When a 20% excess of template was used in annealing primers to templates (the total concentration of annealed primer/template was 50 nM), greater than 95% of the primers could be extended in a time course reaction (data not shown), demonstrating that the majority of primers were annealed to templates under these conditions.

Methods

Steady-State Fluorescence Spectra. Steady-state fluorescence emission spectra were collected from 330 to 460 nm on a SPEX 1681 Fluorolog spectrofluorometer by exciting the sample at 310 nm. Solutions contained 200 nM primer/template, 25 mM HEPES, 50 mM NaCl, and 0.5 mM EDTA at pH 7.5. Concentrations of wild-type and mutant polymerases ranged from 0.6 to 1.2 μM.

Time-Resolved Detection System and Measurements. Time-resolved measurements were performed on 150-μL samples in cuvettes with 3-mm path lengths. A 1-ps 322-nm excitation beam (308-nm excitation for ds DNA alone) was generated from a Coherent (Palo Alto, CA) 702 dye laser, sychronously pumped by a Nd:YAG laser (Coherent Antares). The emission signal was passed through a Glan-Thompson polarizer and 370-nm cut-on filter (340 nm for DNA alone) and a SPEX 0.22-m monochromator set at 375 nm (365 nm for DNA alone) to be detected by a 6 μm microchannel plate detector (Hamamatsu R2809U-01) operating in single-photon-counting, time-correlated mode. The detection electronics comprised a Phillips Scientific 2.5-GHz amplifier (Mahwah, NJ), a Tennelec (Oak Ridge, TN) constant fraction discriminator (TC455) and time-to-amplitude converter (TC862), and a Nucleus pulse-height analysis analog-to-digital converter (MIS3-8K). The stop pulse was obtained from an Antel (Burlington, ON) fast photodiode and constant fraction discrimination (TC455) channel. Typical impulse response functions were 60–80 ps. During acquisitions, the emission polarizer rotated from vertical to horizontal every 30 s (ISS Koalo unit, Urbana, IL). Data sets consist of signal-averaged data from 30–120 30-s acquisitions of vertically or horizontally polarized emissions. Instrument *G* factor (polarization bias) was determined by rotating the excitation beam to the horizontal position and acquiring signal-averaged data for approximately 15 30-s periods for both horizontal and vertical polarized emissions. Fluorescence lifetimes and correlation times were obtained by simultaneous fitting of the vertical and horizontal emission decay curves from each sample using the Globals Unlimited software package (Globals Unlimited, Urbana, IL) (Beechem *et al.*, 1991). The T4 pol tryptophan fluorescence decay was background subtracted from the fluorescence decay of the T4 pol·DNA complex.

Time-Resolved Sample Preparation. All measurements were made in buffers containing 25 mM HEPES, pH 7.5, and 50 mM NaCl. Three separate solutions were prepared containing the following in addition to buffer: (1) 2.9 μM dAPMP; (2) 4 μM pCP/tGT; and (3) 2 μM pCP/tGT, 8 μM T4 pol, and 0.5 mM EDTA.

Stopped-Flow System and Fluorescence Detection. An SFM-3 stopped-flow unit (Molecular Kinetics, Pullman, WA) with an FC.15 cuvette (50-μL volume) and a hard stop shutter was used for stopped-flow reactions. The unit has three stepper-motor-driven syringes. Fluorescence detection for the stopped-flow studies was achieved using a home-built single-photon detector consisting of the following: a Hamamatsu R928 photomultiplier, a 5X 300-MHz amplifier (Stanford Research SR445, Sunnyvale, CA), a discriminator (Stanford Research SR400), and a multichannnel scaler (Tennelec Model MCS-II, Oak Ridge, TN), interfaced to an 80486 microcomputer. The detection system was activated by an external synch-out pulse from a Molecular Kinetic stepper-motor controlling unit. Data acquisition began at least 100 ms before sample mixing. Data was collected using dwell times from 0.25 to 1 ms in 8000 total channels. A 250-W xenon arc lamp (SPEX Fluorolog Model 1681) with fiber optic output directed into the 50-μL sample cell was used for excitation at 310 nm. Fluorescence emission was collected through a 360-nm cut-on filter (Hoya Optics type L36).

Calibration of Stopped-Flow Dead Times. Dead times were determined from the quenching of tryptophan fluorescence by *N*-bromosuccinimide (NBS) (Peterman, 1979) (Bryan Jones and Kenneth A. Johnson, personal communication). Tryptophan fluorescence was excited at 280 nm, with the emission being collected through a 340-nm cut-on filter (Hoya Optics type L34, Fremont, CA). NBS and tryptophan stocks were mixed at a ratio of 1:1 in a total reaction volume of 200 μL. Reactions were performed using 4 μM tryptophan and two different concentrations of NBS (275 and 350 μM). Internal control reactions of tryptophan delivered alone were compared with the initial reaction tryptophan fluorescence to calculate the dead times. Dead time values obtained for deliveries at 6, 8, and 10 mL/s were 8.6 ± 0.8, 7.4 ± 1.0, and 5.2 ± 0.9 ms, respectively.

Association Rates for T4 pol DNA Binding. These measurements were made under conditions similar to those for T4 pol-initiated excision kinetics but by leaving Mg^{2+} out of the reaction buffer. Solutions of primer/template in reaction buffer, T4 pol in reaction buffer, and reaction buffer only were loaded into separate syringes. Reactions were initiated at 20 ± 2 °C by mixing equal volumes (100 or 120 μL) of T4 pol solution with DNA. Reaction mixtures contained final concentrations of 680 nM T4 pol, 200 nM P/T, 25 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM DTT, and 0.1 mM EDTA. Flow rates were 10 mL/s. Multiple runs (from 10 to 16) were summed to increase signal-to-noise. Data were normalized from 0 to 1 using the signals from T4 pol background and 100% reaction. Association rates were calculated from the rate of increase in fluorescence intensity as T4 pol bound AP-labeled DNA by using an adapted bimolecular fitting routine of the Globals analysis software (Globals Unlimited; Urbana, IL.).

Stopped-Flow Single-Turnover Excision Reactions Initiated by Addition of T4 Polymerase. Solutions of primer/template in reaction buffer, T4 pol in reaction buffer, and reaction buffer alone were each placed in a separate syringe. Reactions were initiated by mixing equal volumes (120 μL) of a solution of T4 pol and a solution of primer/template. Reactions were performed at 20 ± 2 °C, and reaction mixtures contained final concentrations of 680 nM T4 pol, 200 nM primer/template, 25 mM HEPES, pH 7.5, 50 mM NaCl, 7 mM $MgCl_2$, and 1 mM DTT. Multiple runs (10–15) of the same reaction were summed to increase signal-to-noise. Flow rates for reactions ranged from 6 to 8 mL/s. Background measurements were made by mixing reaction buffer with primer/template only and with T4 pol only. Since T4 pol

contributed most to the background, data were normalized from 0 to 1 using the signals for T4 pol background and 100% reaction. Increases in fluorescence were fit to the sum of one or two exponentials and a constant as in eq 1, where $k_1 = 1/\tau_1$ and $k_2 = 1/\tau_2$. The nonlinear regression routine in SigmaPlot 4.1 (Jandel Scientific) or Globals Unlimited (Urbana, IL) was used to fit data.

$$y = a_1(1 - e^{-t/\tau_1}) + a_2(1 - e^{-t/\tau_2}) + c \quad (1)$$

Stopped-Flow Anisotropy Measurements of T4 pol-Initiated Excision Reactions. A T-format steady-state anisotropy fluorometer was assembled with the following components: A 306-nm vertically polarized excitation beam was provided by a Coherent (Palo Alto, CA) 702 dye laser, pumped by a Nd:YAG laser (Coherent Antares). The beam was focused onto an SFM3 stopped-flow (as above) cuvette. Sample mixing was done at 8 mL/s. On each side of the SFM3 syringe unit a homemade emission channel platform was attached which allowed two emission channels to be aligned on the cuvette at one time. Each of the two detection channels was composed of an ISS emission channel (Urbana, IL) with a Glan Thompson polarizer and a 360-nm cut-on filter (Hoya Optics type L36). A Hamamatsu R928 photomultiplier tube (PMT) was mounted on the end of each emission channel. The signal from each PMT was then amplified through an SR445 DC-300 amplifier (Stanford Research) before being discriminated with an SR400 (Stanford Research) two-channel photon counter. Output from the SR400 was detected by an MCS-II multichannnel scalar card (Tennelec Nucleus) within a 486DX microcomputer. The MCS-II card was synchronized with the SFM3 stopped-flow unit through the external synch output from the SFM3. The time base for the experiment was 250 μs. Reactions were performed by mixing equal volumes (100 μL) of T4 pol and DNA. Multiple kinetic runs were summed to obtain adequate signal-to-noise. Final reaction mixtures contained 50 mM NaCl, 25 mM HEPES, pH 7.5, 0.25 mM EDTA, 8 mM $MgCl_2$, and 1 mM DTT. Reaction mixtures with double-stranded DNA contained 200 nM pAP/tTT and 400 nM T4 pol and for single-stranded DNA contained 200 nM pTP or pAP and 680 nM T4 pol. The anisotropy function is defined as

$$r(t) = \frac{I_\parallel G - I_\perp}{I_\parallel G + 2I_\perp} \quad (2)$$

where $I_\parallel$ and $I_\perp$ are the polarized fluorescence emission parallel and perpendicular to the excitation beam, respectively. An instrument polarization bias (G factor) was measured using horizontally polarized excitation and varied from 0.8 to 1.2 depending upon the experiment. Results from the anisotropy experiments were fit as exponentials.

RESULTS

Fluorescence of 2-Aminopurine. The excitation maximum of dAPMP is at 304 nm, and the emission maximum is at 368 nm. When AP is present in solution as the free monophosphate, its fluorescence is about 25–125 times as intense as when the nucleotide is incorporated at the primer 3′-terminus of a ds P/T (Figure 1a). The fluorescence intensity of AP in DNA is sequence dependent, varying by as much as a factor of 4 for a 5′-nearest neighbor T in comparison with G (Bloom *et al.*, 1993). In single-stranded DNA the fluorescence intensity is not quenched as much as in double-stranded DNA (Figure 1a). This signal difference between AP as a free nucleotide

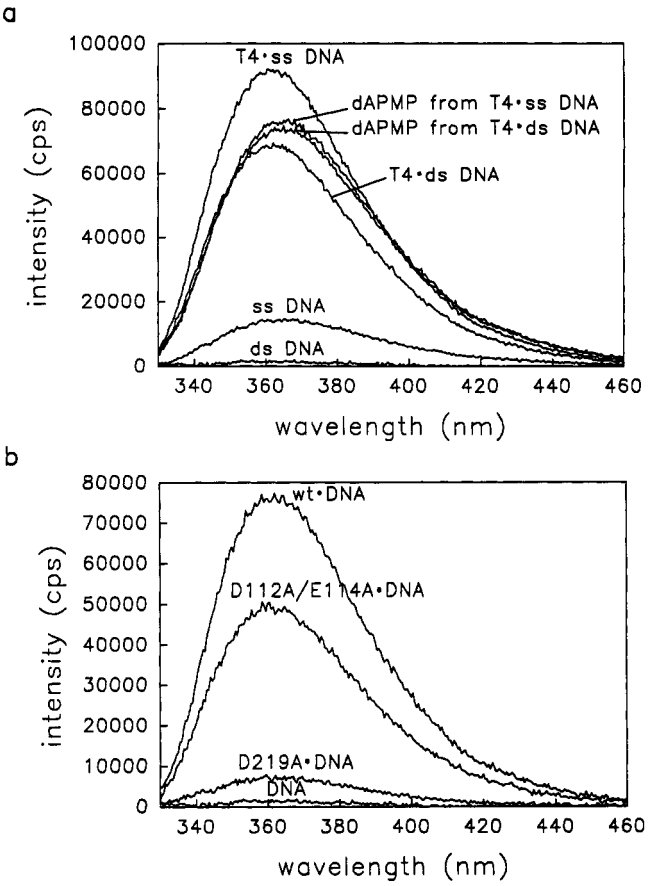

FIGURE 1: Fluorescence emission spectra for 2-aminopurine in different environments. (a) Emission spectra for a complex between T4 pol (680 nM) and 200 nM single-stranded pCP in the presence of EDTA and the absence of Mg^{2+} (T4•ss DNA), free 2-aminopurine deoxyribonucleoside 5′-monophosphate excised from the T4 pol•pCP complex when Mg^{2+} was added (dAPMP from T4•ss DNA), a complex between T4 pol (680 nM) and 200 nM double-stranded pCP/tGT (T4•ds DNA), free 2-aminopurine deoxyribonucleoside 5′-monophosphate excised from the T4 pol•pCP/tGT complex after Mg^{2+} was added (dAPMP from T4•ds DNA), 200 nM single-stranded pCP alone (ss DNA), and 200 nM double-stranded pCP/tGT alone (ds DNA). (b) Emission spectra for polymerase•DNA complexes of wild-type T4 pol (600 nM) with 200 nM pCP/tGT (wt•DNA), of exonuclease-deficient mutant D112A/E114A (1100 nM) with 200 nM pCP/tGT (D112A/E114A•DNA), and of exonuclease-deficient mutant D219A (600 nM) with 200 nM pCP/tGT (D219A•DNA), and for 200 nM pCP/tGT alone (DNA).

and AP incorporated in DNA can be used to follow the kinetics of both nucleotide insertion and excision.

The fluorescence of AP at the primer 3′-terminus of ds DNA is enhanced on binding to T4 pol in the absence of Mg^{2+} and in the presence of EDTA, conditions which inhibit the exonuclease (Figure 1a). The fluorescence of a bound complex between T4 pol and ss DNA is even greater than that of free dAPMP. The emission maxima of the complexes (363 nm) are blue shifted relative to that of the DNA P/T alone (368 nm). Complexes between T4 pol and ds DNA are less fluorescent than complexes with ss DNA probably because stronger AP stacking in ds DNA increases the ratio of annealed to melted out termini. The difference in fluorescence in the bound complexes might reflect the relative partitioning of the DNA between the polymerase and exonuclease active sites.

An observation possibly related to the relative partitioning of the binding of DNA in either the exonuclease or the polymerase active site of T4 pol was made for complexes between wild-type T4 pol and two different exonuclease-deficient mutants of T4 pol and ds P/Ts with AP at the primer

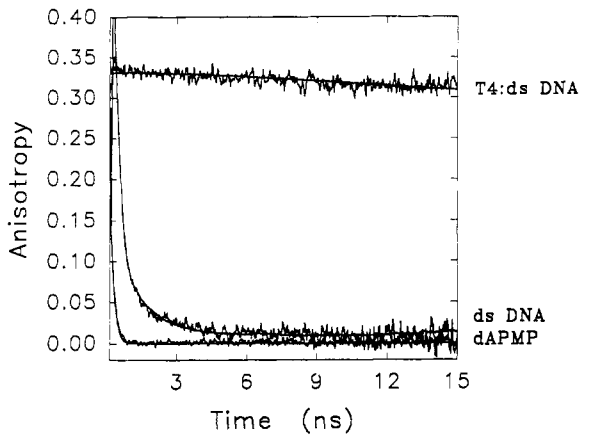

FIGURE 2: Time-resolved anisotropy decays for 2-aminopurine in different environments. Anisotropy decays are shown for solutions containing 2 μM pCP/tGT and 8 μM T4 pol (T4·ds DNA), 4 μM pCP/tGT (ds DNA), and 2.9 μM dAPMP (dAPMP). AP was excited at 322 nm, and emission was measured using a 370-nm cut-on filter for the T4·ds DNA complex and dAPMP. AP was excited at 306 nm, and emission was monitored using a 340-nm cut-on filter for ds DNA.

3′-terminus. One mutant has a single mutation, D219A, and the other has two mutations, D112A and E114A (Reha-Krantz & Nonay, 1993). The intensities of AP fluorescence for complexes between these exonuclease-deficient mutants and ds P/Ts are lower than those for complexes with the wild-type enzyme (Figure 1b). Perhaps binding of the primer terminus in the wild-type exonuclease active site results in an increase in fluorescence by decreasing base-stacking interactions and thereby enhancing melting of the primer terminus. For each mutant, the P/T was titrated with enzyme until the DNA was saturated and the fluorescence intensity reached a maximum. The exonuclease-deficient mutant D219A has been shown to have the same K_D for DNA as wild type (Frey *et al.*, 1993; Reha-Krantz & Nonay, 1993). Thus, the decrease in fluorescence intensity of complexes between the mutants and DNA does not arise from substoichiometric binding of the DNA.

The localization of AP in different environments can be characterized by time-resolved decays of fluorescence anisotropy. The time-resolved fluorescence anisotropies of free dAPMP, dAPMP within duplex DNA (pGP/tGT), and bound T4 pol·pGP/tGT complex are shown in Figure 2. The free dAPMP rotates very rapidly (rotational correlation time, ϕ, = 0.123 ns), as does the AP located at the end of the primer/template (ϕ_1 = 0.129 ns; ϕ_2 = 1.13 ns). However, the T4 pol·DNA bound complex reveals a much slower rotation of the AP (ϕ = 98.2 ns) with essentially no local mobility. Observation of this bound T4 DNA complex in the absence of Mg^{2+} confirms that the large increase in fluorescence intensity for this complex (Figure 1a) does not arise from formation of free dAPMP. These bound complexes will be described fully in a future study (M. R. Otto *et al.*, unpublished results).

Polymerase·DNA Association Rates. The sequence-dependent differences in excision rates do not arise from a rate-determining polymerase·DNA association step. The observed rates for the increase in fluorescence in exonuclease reactions initiated by adding T4 pol to DNA are independent of the concentration of T4 pol when the concentration of T4 pol is in excess of the DNA concentration (data not shown).

T4 pol·DNA association rates were measured under conditions identical to conditions for exonuclease reactions but in the absence of Mg^{2+}. No exonuclease reaction occurs under these conditions, which was verified by measuring the time-resolved anisotropy decay for the complexes. The rate of association was measured by following the increase in AP fluorescence as T4 bound P/Ts. Time courses were fit to single exponentials. The observed rates were 108, 123, 104, and 71 s^{-1} for primer/templates pCP/tGT, pAP/tTT, pGP/tCT, and pTP/tAT, respectively, in reaction mixtures containing 680 nM T4 pol and 200 nM P/T (data not shown). These rates correspond to bimolecular association rates of 2–4 $\times$ 10^8 M^{-1} s^{-1} and are much faster than observed rates for excision. These rates are similar to those reported by Capson *et al.* (1992).

Kinetics of Exonucleolytic Removal of AP Initiated by T4 pol Addition. Pre-steady-state kinetics of removal of AP from P/Ts of different sequences were measured using stopped-flow fluorescence techniques to follow the increase in fluorescence as AP was excised. Reaction mixtures contained an excess of T4 pol over P/T and were the result of a single turnover of substrates to products. Reactions were initiated by the addition of a solution of T4 pol and Mg^{2+} to a solution of P/T and Mg^{2+} at 20 °C as described in Methods. Primer/templates shown in Table 1 will be referred to using the nomenclature indicated. Fluorescence emission spectra and time-resolved anisotropies of reaction products are indistinguishable from those of free dAPMP.

Primer/Templates with Different AP·X Base Pairs. Exonucleolytic removal of AP when paired opposite T, A, C, or G was measured using primers of identical sequence (pCP in Table 1). Time courses for these reactions are shown in Figure 3a. Removal of AP is slowest when it forms a correct pair with T. The overall rate of removal of AP from AP·X base pairs increases as T < A < C < G. For AP·A, AP·C, and AP·G pairs, reaction time courses appear to be biphasic. A much better fit to the data at early reaction times is obtained using a double exponential rather than a single exponential (Figure 3b). Plots of residuals for a single-exponential fit of AP·T data and residuals for both single- and double-exponential fits of AP·G data are given in Figure 3c and illustrate the quality of the two fits. Residuals for the single-exponential fit of AP·G data are not randomly distributed about 0 at early reaction times, while a more random distribution was observed for the double-exponential fit. On the basis of the same analysis, removal rates of dAPMP from AP·C and AP·A mispairs are described more accurately using double- rather than single-exponential fits.

For stopped-flow reactions, there is a small time delay, the dead time, between the time at which the reactants are mixed and the time at which data acquisition begins. If dead times and reaction rates are known, the fraction of a reaction that will be missed before data acquisition begins can be calculated. Conversely, dead times for a system can be calculated from the fraction of reaction missed when data acquisition begins and the rate of the reaction. For single-exponential fits of excision of AP from mispairs, calculated dead times, based on the fraction of reaction missed when data acquisition began, are much longer (23–39 ms) than those that were determined experimentally (5–9 ms; data not shown) using a standard reaction (see Methods). Double-exponential fits which include an early rapid phase are consistent with measured dead times. When data are fit by including experimentally determined dead times, the calculated amplitude of the rapid phase increases and the value of the dead time constant, c (Table 2), decreases. For the correct AP·T pair, none of the reaction was missed before data acquisition began, and a single exponential provides an excellent fit to the data.

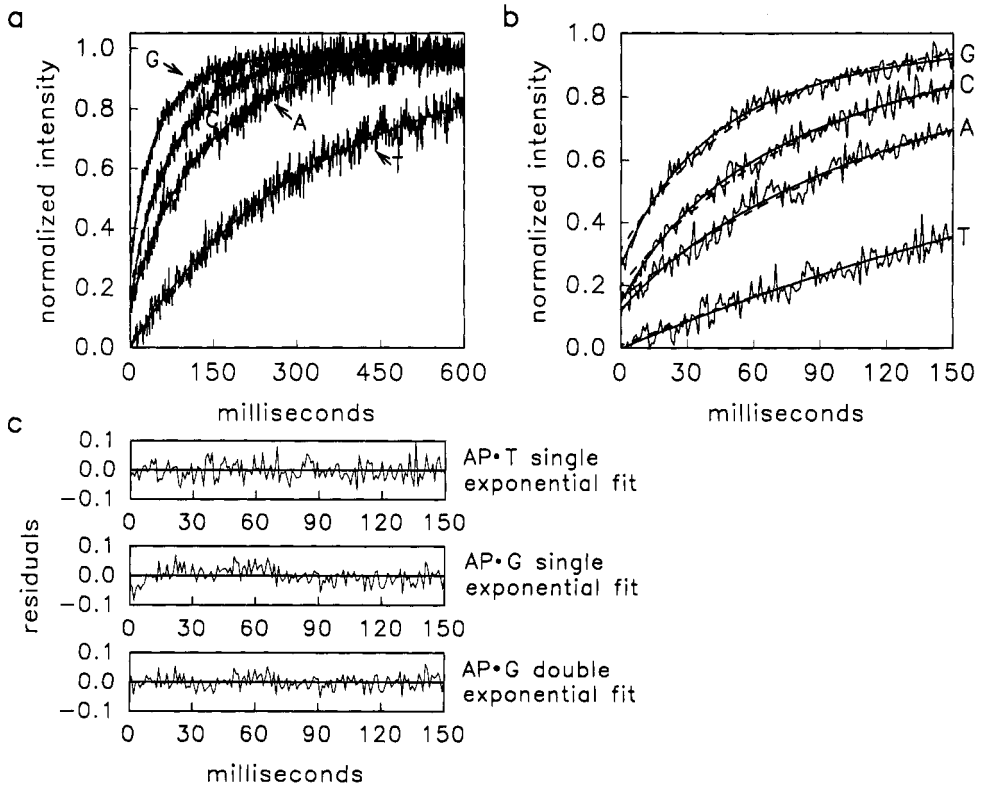

FIGURE 3: Time courses for excision of dAPMP from primer/templates with different AP•X base pairs. (a) Time courses for excision of AP from AP•T (T), AP•A (A), AP•C (C), and AP•G (G) base pairs in reactions initiated by the addition of a solution of T4 pol and Mg^{2+} to a solution of DNA and Mg^{2+} at 20 °C as described in Methods. Reaction mixtures contained 680 nM T4 pol and 200 nM DNA [pCP/tGT (T), pCP/tGA (A), pCP/tGC (C), and pCP/tGG (G); (see Table 1 for sequences)]. (b) The same data as in (a) but at early reaction times. The dashed lines show single-exponential fits to the data, and the solid lines show double-exponential fits. (c) Residuals for a single exponential fit to data for an AP•T pair and for single- and double-exponential fits to data for an AP•G pair.

Table 2: Single-Turnover Kinetics of Excision of 2-Aminopurine (AP) from Primer 3′-Termini by T4 DNA pol in Reactions Initiated by T4 pol Addition[a]

P/T	5′-neighbor(s)	base pair	a_1[b]	k_1 (s^{-1})[c]	a_2	k_2 (s^{-1})	c[d]
pCP/tGT	C	AP•T[e]			0.97 (1.1)	2.9 (2.2)	0.0079 (–0.059)
pCP/tGA	C	AP•A[e]	0.089 (0.073)	33 (42)	0.78 (0.77)	6.5 (5.5)	0.12 (0.15)
pCP/tGC	C	AP•C[e]	0.18 (0.16)	40 (32)	0.66 (0.66)	9.8 (7.6)	0.14 (0.17)
pCP/tGG	C	AP•G[e]	0.31 (0.39)	40 (30)	0.42 (0.37)	13 (7.8)	0.26 (0.21)
pGP/tCT	G	AP•T			0.91	6.8	0.068
pTP/tAT	T	AP•T			0.87	7.7	0.13
pAT/tTT	A	AP•T			0.76	10	0.25
pGC/tGC_T	CGGC	AP•T[f]	0.097 (0.13)	37 (53)	0.82 (0.62)	4.3 (4.3)	0.067 (0.22)
pGC/tGC_C	CGGC	AP•C[f]	0.20 (0.39)	38 (23)	0.58 (0.31)	9.1 (6.6)	0.21 (0.33)
pAT/tAT_T	TAAT	AP•T[f]	0.55 (0.25)	37 (26)			0.44 (0.72)
pAT/tAT_C	TAAT	AP•C[f]	0.48 (0.19)	42 (38)			0.51 (0.73)

[a] Single-turnover reactions were initiated by the addition of T4 pol to DNA at 20 °C as described in Methods. Final concentrations of T4 pol and DNA were 680 and 200 nM, respectively. Primer/template sequences are shown in Table 1. [b] The amplitudes of the rapid and slow phases are given as a_1 and a_2, respectively. [c] The observed rates of the rapid and slow phases are given by k_1 and k_2, respectively. [d] The fraction of reaction missed during the mixing dead time is given by c. [e] Values in parentheses for the AP•X data set are for a separate experiment using a different preparation of T4 pol and a smaller 9-μL cuvette which reduces dead time. Final concentrations of DNA and T4 pol were 200 and 500 nM, respectively. [f] Values in parentheses for the data set with different G•C versus A•T content are for a separate set of experiments under identical conditions.

Biphasic kinetics data indicate that two kinetic processes are occurring. A fit of these data yields both rate (k_1 and k_2; Table 2) and associated amplitude (a_1 and a_2; Table 2) terms for each phase of the reaction. The fraction of a reaction that is proceeding by a given pathway is related to the relative amplitude of that phase of the reaction. Observed rate constants, k_1, for the rapid phases are independent of DNA sequence with an average rate of 37 ± 7 s^{-1} for all reactions in Table 2. The rates of the slower phases, k_2, are sequence dependent, varying by as much as a factor of 4 and increasing as AP•T < AP•A < AP•C < AP•G. As the nucleotide opposite AP is changed from A to C to G, the amplitude of the rapid phase increases, indicating that the kinetic partitioning between the two pathways is also sequence dependent. The decrease in the amplitude of the slower phase is correlated with the increase in the rate of this phase and the increase in the amplitude of the rapid phase. This correlation suggests that the rate of the slow phase is the rate of conversion to a species that reacts rapidly. An increase in the rate of this conversion would shift the equilibrium to favor the rapidly reacting species and thus decrease the relative amplitude of the slow phase (see Discussion, T4-Initiated Excision of AP from Primer/Templates; Figure 8).

Primer/Templates with Different 5′-Neighbors to AP. Pre-steady-state kinetics of removal of AP were measured for P/Ts of identical sequence except for the base pair immediately 5′ to AP (Table 1). These sequences are also identical to those used to measure excision of AP from mispairs. In each

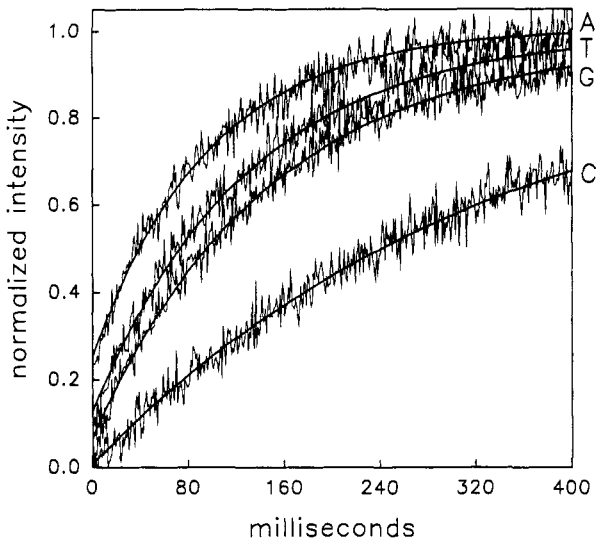

FIGURE 4: Time courses for excision of AP from primer/templates with different 5′-neighbors to AP. Reactions were initiated by the addition of T4 pol to DNA, and reaction mixtures contained 680 nM T4 pol and 200 nM DNA at 20 °C as described in Methods. Primer/templates contained C pCP/tGT (C), G pGP/tCT (G), T pTP/tAT (A), and A pAP/tTT (T) on the primer immediately 5′ to AP. The smooth lines show single-exponential fits of the data.

case AP forms a correct base pair with T. Changing the base pair immediately 5′ to an AP·T base pair affected the rate of removal of AP, resulting in an increase with 5′-neighbor to AP as C < G < T < A (Figure 4). These data for removal of AP from correct AP·T pairs are very nearly fit by a single exponential. A small component of a rapid phase may be present at early times for primer/templates with G, T, and A neighbors as indicated by the small deviation of the data from the calculated single-exponential fit within the first 20 ms of reaction. The relatively small difference in the rates of the two phases (the slow rates given by k_2 are a factor of 4–5 slower than the rapid rate of ~37 s^{-1} given by k_1) and the small amplitude of the rapid phase make the presence of this phase difficult to establish for correct pairs within this sequence context.

When these reactions were initiated by the addition of Mg^{2+} to a preformed T4 pol·DNA complex, reaction time courses showed the presence of an additional phase (data not shown). Initially, a T4 pol·DNA complex that was more fluorescent than DNA alone (Figure 1a) was present. Addition of Mg^{2+} to this complex resulted in a rapid decrease in fluorescence (140–250 s^{-1}) followed by a slow increase in fluorescence at rates equal to those measured in the T4 pol-initiated reactions described above. The additional phase which gives a rapid decrease in fluorescence could be due to a change in the binding environment of AP from a more fluorescent to a less fluorescent enzyme·DNA complex or to rapid removal of dAPMP from a hyperfluorescent enzyme·DNA complex which is more fluorescent than free dAPMP. Investigations into the nature of the early Mg^{2+} phase will be described in a future study.

Primer/Templates Which Vary in G·C Content. Pre-steady-state kinetics of removal of AP were measured for four primer/templates of identical sequence except for the four base pairs immediately 5′ to AP and the nucleotide base opposite AP. For one pair of P/Ts, four G·C base pairs are immediately 5′ to either an AP·T or an AP·C base pair, and for the other pair four A·T base pairs are immediately 5′ to an AP·T or an AP·C pair (Table 1). The entire sequence for each of these P/Ts is different from those used to measure excision from different AP pairs and next to different 5′-neighbors. The

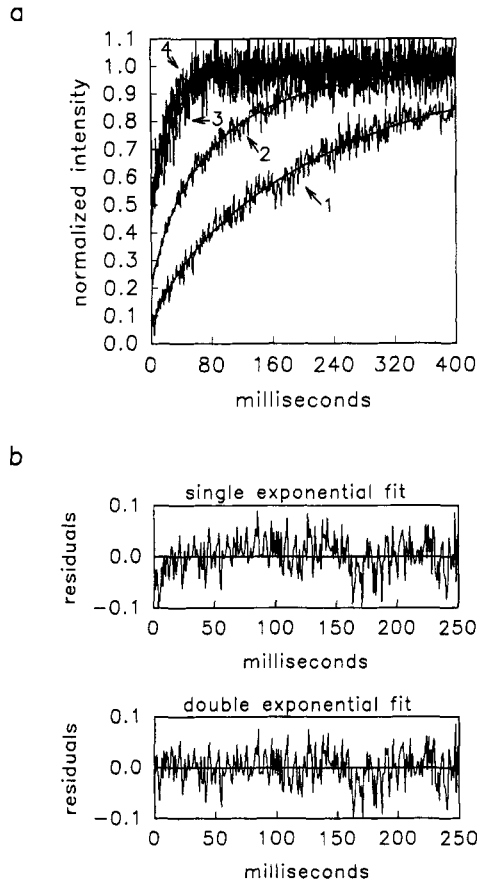

FIGURE 5: (a) Time courses for excision of AP from AP·T or AP·C base pairs in primer/templates which differ in G·C content. Reactions were initiated by the addition of a solution of T4 pol and Mg^{2+} to a solution of DNA and Mg^{2+} at 20 °C as described in Methods. Time courses show the excision of AP from AP·T of pGC/tGC_T (1), from AP·C of pGC/tGC_C (2), from AP·T of pAT/tAT_T (3), and from AP·C of pAT/tAT_C (4) in reaction mixtures containing 680 nM T4 pol and 200 nM P/T. Smooth lines show double-exponential fits to data sets 1 and 2 and single-exponential fits to data sets 3 and 4. (b) Residuals for single- and double-exponential fits of data for excision of AP from AP·T of pGC/tGC_T.

presence of G·C- or A·T-rich sequences 5′ to AP dramatically affects the rate of removal of AP. AP is excised the slowest when paired opposite T in the P/T containing four G·C pairs, and it is excised the fastest when paired opposite T or C in the P/T containing four A·T pairs (Figure 5a). An important result is that a correct AP·T pair in the A·T-rich P/T is removed about 4 times faster than an AP·C mispair in the G·C-rich P/T (Figure 5a). This result is opposite that shown earlier (Figure 3a) where an AP·C mispair was removed faster than an AP·T correct pair within the *same* sequence context. The reversal in rates of excision of AP·T versus AP·C suggests that a change in the local DNA sequence can alter the discrimination of the exonuclease between correct (AP·T) and incorrect (AP·C) base pairs. Another significant result is that, for the A·T-rich P/Ts, the exonuclease removes AP from a correct AP·T pair and an AP·C mispair at similar rates. Within this sequence context, the exonuclease shows no discrimination between the correct pair and the mispair.

Time courses for the slower reactions for the two P/Ts containing four G·C base pairs were fit to double exponentials. Calculated residuals for removal of AP opposite T in a G·C-rich sequence are given in Figure 5b. Time courses for the two faster P/Ts containing four A·T base pairs were fit to single exponentials. Observed rate constants and amplitudes are given in Table 2. For the two G·C P/Ts, the rates of the

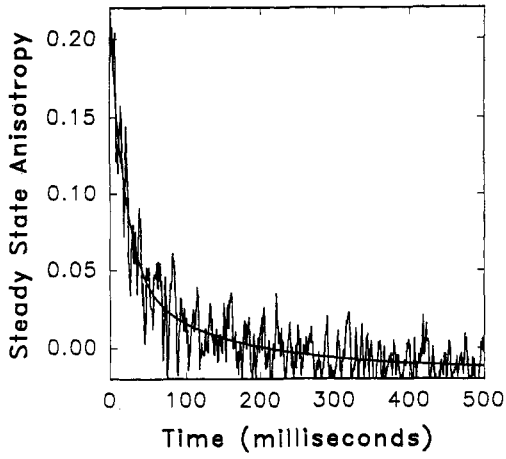

FIGURE 6: Decrease in anisotropy over the time course of excision of AP from an AP·T base pair in pAP/tTT in a reaction mixture containing 400 nM T4 pol and 200 nM DNA as described in Methods.

rapid phases are similar but the amplitude increases when going from an AP·T to an AP·C pair. The rate of the slow phase increases, while its amplitude decreases, when going from AP·T to AP·C. These results are consistent with the results above for AP·T and AP·C base pairs within the same sequence context. For the two A·T P/Ts, only the rapid phase is present; there is no slower sequence-dependent phase.

Anisotropy and Polarization Effects. A T4 pol·DNA complex has a large anisotropy ($r \approx 0.25$), while the anisotropy of free dAPMP is approximately zero. The anisotropy of the AP-labeled DNA is closer to that of free dAPMP than to that of the T4 pol·DNA complex (Figure 2). During the time course of an excision reaction, there is a large change in the anisotropy of AP as it changes environments from the enzyme·DNA complex to the free nucleotide in solution. In addition to measuring the increase in intensity of AP as it was excised, we measured the decrease in the steady-state anisotropy during the excision reaction. Figure 6 shows the decrease in anisotropy over the time course of a reaction for removal of dAPMP when paired opposite T in primer/template pAP/tTT by T4 pol. Analysis of the total fluorescence intensity calculated from the anisotropy ($I_{\parallel} + 2I_{\perp}$) over the time course of the reaction yields a rate constant (k_2 = 9.2 s^{-1}) which is in good agreement with the value obtained from direct measurement (without polarizers) of intensities for the excision reaction (k_2 = 10 s^{-1}; Table 2). Rigorous analysis of combined total intensity and anisotropy data (which include terms accounting for the quantum yield) demonstrate that the reaction is biphasic. The very rapid increase in total intensity is associated with a very rapid decrease in anisotropy.

When fluorescence intensity measurements are made in the absence of polarizers, changes in intensity may be observed which originate only from changes in the polarization of the emitted fluorescence (Weber & Teale, 1957). Generally, while these polarization terms have not been taken into account in stopped-flow intensity studies, they can be eliminated by performing stopped-flow experiments under "magic angle" conditions [see, e.g., Badea and Brand (1979)]. Alternatively, given a steady-state anisotropy value, one can correct the observed intensity as described previously (Cehelnik *et al.*, 1975; Mielenz, 1982). In this study, both magic angle total intensity and anisotropy data were collected on selected primer/templates (e.g., Figure 6) to determine whether the rapid phases observed in fluorescence intensity measurements of excision kinetics on some primer/templates could have been caused by polarization bias instead of an actual reaction. We determined that polarization bias had a negligible effect on the intensities observed, leading us to conclude that the rapid kinetic phase is attributable to release of dAPMP.

Excision of 2-Aminopurine from Single-Stranded DNA. Steady-state anisotropy experiments where AP is removed from the 3′-terminus of single-stranded DNA show a single kinetic phase with rate constants of 80 and 65 s^{-1} for pTP and pAP, respectively (data not shown). These reactions were initiated by addition of Mg^{2+} to a solution of T4 pol and single-stranded DNA. These rates are consistent with the rate of 100 s^{-1} measured previously for the removal of a natural nucleotide from single-stranded DNA (Capson *et al.*, 1992). The excision rate for ss DNA most likely represents the maximum rate for the exonuclease in the absence of any effects of DNA structure or strand melting (Donlin *et al.*, 1991; Capson *et al.*, 1992).

DISCUSSION

Influence of Local DNA Sequence on Excision of AP. Variations in local DNA sequence in the region of the primer 3′-terminus have a dramatic effect on the rates of excision of AP by T4 DNA polymerase. X-ray crystal structure (Freemont *et al.*, 1988; Beese *et al.*, 1993) and cross-linking studies (Cowart *et al.*, 1989) with Klenow fragment suggest that at least a four- or five-nucleotide segment of the primer terminus must dissociate from the template in order for the primer terminus to be accessible to the exonuclease active cleft. Cross-linking studies for T4 pol (Cowart *et al.*, 1989) suggest that only two base pairs are required to melt for the exonuclease to be active on the primer terminus. In single-turnover experiments, T4 pol was found to remove a terminal mismatch in addition to the next correctly paired nucleotide immediately 5′ to the terminal nucleotide without dissociating (Reddy *et al.*, 1992). These data also suggest that the exonuclease site accommodates two nucleotides at the primer 3′-end. The observed effects of local DNA sequence on the efficiency of proofreading by T4 DNA polymerase are consistent with the idea that a primer terminus must dissociate from the template in order to be a substrate for the exonuclease as suggested previously (Brutlag & Kornberg, 1972; Muzyczka *et al.*, 1972; Bessman & Reha-Krantz, 1977; Clayton *et al.*, 1979; Brenowitz *et al.*, 1991). Local DNA sequences that tend to stabilize the region around the primer terminus and make local melting more difficult lead to slower excision of dAPMP. Those that tend to destabilize the region lead to more rapid hydrolysis.

AP is removed fastest when it forms a mispair with G and slowest when it forms a correct pair with T. The ease of removal of AP from AP·X pairs is correlated with the reduced stabilities, as judged by T_m measurements, of duplexes containing these base pairs. Melting temperatures for four heptamer duplexes that were identical except for the central AP·X base pair were 31.4, 28.0, 24.9, and 20.4 °C for duplexes containing AP·T, AP·A, AP·C, and AP·G pairs, respectively (Erijta *et al.*, 1986). Since the nucleotide paired opposite AP is the only difference in these duplexes, the differences in T_m's largely reflect differences that the AP·X base pair contributes to the stability of the duplex. It is interesting to note that the AP·A wobble base pair (Fazakerley *et al.*, 1987) is more stable than the AP·C pair, which is likely to exist predominantly as a protonated Watson–Crick base pair (Sowers *et al.*, 1986) under our experimental conditions. Although the AP·A base pair is more stable than an AP·C pair within a duplex, a polymerase makes an AP·C mispair more readily than a AP·A mispair (Mhaskar & Goodman, 1984). This may reflect

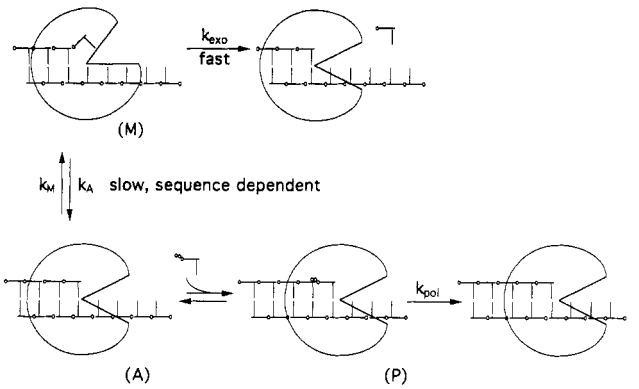

FIGURE 7: Model for DNA sequence dependent excision by T4 DNA polymerase. The selectivity of the exonuclease of T4 pol may rely on the recognition of the geometry of the base pair at a primer 3′-terminus. The primer terminus may adopt two conformations: one where the terminal base pair is in an annealed structure (A state) and one where the terminal base pair is in a melted-out structure (M state). The T4 pol is illustrated by a wedged circle, in either of two conformations. In the polymerase conformation, annealed correct or incorrect base pairs are bound in the polymerase active site, states (A) and (P); in the exonuclease conformation, melted correct or incorrect base pairs are bound in the exonuclease active site, state (M). The slow, sequence-dependent step may reflect the rate of switching from the polymerase to the exonuclease active site. Since a mispair is incapable of forming a normal Watson–Crick type base pair, it is more likely to exist in the M state than a correct pair and thus more likely to be excised. Correct pairs may also exist in either a melted-out state, where excision can take place, or an annealed state, although an annealed state is likely to be strongly favored. Local DNA sequences that are predicted to stabilize a DNA duplex would also be expected to increase the relative population of the A state for both correct and incorrect pairs and thus decrease the efficiency of proofreading.

geometric constraints within the polymerase active site. The opposite selectivity is observed for the exonuclease reaction. The exonuclease of T4 DNA polymerase removes the less stable AP·C base pair more easily than the AP·A pair.

A G·C-rich DNA sequence in general will be higher melting and more stable than an A·T-rich sequence. The presence of four G·C base pairs immediately 5′ to AP reduced the efficiency of removal of AP by T4 polymerase in comparison to P/Ts of identical sequence but with four A·T pairs immediately 5′ to AP (Figure 5a). Again, T4 polymerase excised a nucleotide more readily from a DNA sequence context predicted to be less stable. For primer/templates of identical sequence except for the AP·X base pair (discussed above), AP was removed more rapidly from an AP·C mispair than from an AP·T correct pair. However, when an AP·C mispair was placed in a sequence containing four G·C pairs 5′ to AP (pGC/tGC_C), it was removed less rapidly than an AP·T correct pair in a P/T containing four A·T pairs 5′ to AP (pAT/tAT_T). For the AP·C pair in the G·C-rich sequence about one-quarter of dAPMPs were excised in a rapid reaction ($k_1 = 37\ s^{-1}$), while the remaining three-quarters were removed at a slower rate ($k_2 = 9.1\ s^{-1}$); for the AP·T pair in the A·T-rich sequence all of the dAPMP was excised in a rapid reaction ($k_1 = 37\ s^{-1}$). This result demonstrates that the selectivity of the exonuclease is dependent not only on the identity of the terminal base pair but also on the local DNA sequence context.

These data support the idea that mutations are more likely to occur in G·C-rich compared to A·T-rich sequence contexts (Bessman & Reha-Krantz, 1977). The polymerization and proofreading exonucleolytic reactions compete during DNA synthesis, with excision favored at melted primer termini and continued polymerization favored at annealed termini (Figure 7). If stabilization of mispairs occurs in G·C-rich sequences, then proofreading may be relatively less efficient, leading to an increase in mutations. Conversely, in the absence of stabilization in A·T-rich sequences, mispairs are excised with relatively high efficiency, resulting in fewer mutations. The apparent lack of exonuclease selectivity in the removal of AP·C and AP·T base pairs in A·T-rich regions implies that a significant fraction of correct base pairs are also excised (Clayton *et al.*, 1979). Thus, DNA synthesis is predicted to occur with high fidelity in A·T-rich sequences, accompanied by the elevated "cost" associated with the removal of as many as 5–15% of correctly inserted nucleotides (Clayton *et al.*, 1979; Fersht *et al.*, 1982).

The removal of AP opposite T by T4 DNA polymerase was sensitive to the base pair immediately 5′ to AP. Rates of removal of AP varied by a factor of 3 when the 5′-neighbor was changed from C to A (Table 2). These rates do not seem to depend on whether a purine or a pyrimidine base-stacking partner is 5′ to AP. The selectivity may depend on whether a G·C or an A·T base pair is immediately 5′ to AP.

Wild-Type and Mutant T4 pol Complexes with DNA in the Absence of Mg^{2+}. The fluorescence intensity of AP-labeled DNA increased dramatically when the DNA was bound to T4 pol in the absence of Mg^{2+}. We have observed that the fluorescence intensity of AP increases on going from double-stranded DNA to single-stranded DNA to free nucleotide. Thus, any changes that reduce interactions between neighboring nucleotides and AP in aqueous solution result in an increase in fluorescence of AP.

A bound complex between T4 pol and AP-labeled DNA leads to an increase in fluorescence. One component of the fluorescence increase is attributable primarily to the bound enzyme, because the increased fluorescence on going from free ss DNA to the bound enzyme state (T4·ss DNA; Figure 1a) is greater than that of either free ss DNA or free dAPMP (Figure 1a). A second component contributing to an increase in fluorescence in the polymerase bound complex can arise from the effect of the enzyme on the stability of the primer terminus. The fluorescence intensity of a polymerase complex with single-stranded DNA is greater than that with double-stranded DNA, probably because ds DNA can bind either in a melted-out single-stranded-like conformation or in an annealed conformation, where stacking interactions are stronger. Since T4 pol is more active on single- than on double-stranded DNA, decreased stacking may stimulate more rapid enzymatic hydrolysis of AP.

The fluorescence intensity of AP may be different for primer termini bound in the polymerase and exonuclease active sites. An increase in enzyme bound fluorescence intensity could reflect a higher proportion of ss DNA bound in the exonuclease, as opposed to the polymerase, active site. These two possibilities are not mutually exclusive, however because binding in the exonuclease active site may cause destabilization (melting) of the primer terminus, while binding in the polymerase active site may encourage stabilization (annealing) of the primer terminus (Figure 7).

Complexes between AP-labeled DNA and two exonuclease-deficient mutants of T4 pol (D219A and D112A/E114A) were less fluorescent than the corresponding complexes with wild-type polymerase. The exonuclease activity of these mutants may be reduced because they are unable to bind DNA in the exonuclease site or in the proper geometry possibly in a melted-out state. This inability to bind DNA in the proper geometry or in the exonuclease site may also result in the decreased fluorescence observed for the mutant·DNA complexes. These results should be viewed with caution, however,

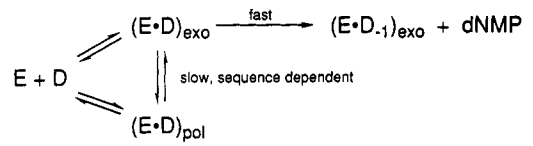

FIGURE 8: Model for excision by T4 DNA polymerase. T4 pol binds DNA in two states: one which is active for hydrolysis of the 3′-terminal nucleotide, $(E\cdot D)_{exo}$, and one which is inactive for hydrolysis, $(E\cdot D)_{pol}$. The active complex, $(E\cdot D)_{exo}$, reacts in a rapid reaction. The inactive complex, $(E\cdot D)_{pol}$, undergoes a slow conversion to the active complex where it reacts rapidly. The relative populations of the $(E\cdot D)_{exo}$ and $(E\cdot D)_{pol}$ complexes and the rate of conversion from the $(E\cdot D)_{pol}$ to the $(E\cdot D)_{exo}$ state are dependent on DNA sequence. It is possible that the active $(E\cdot D)_{exo}$ state is a complex where the DNA is bound at the active site of the exonuclease or in a conformation where the primer terminus is melted out. The inactive $(E\cdot D)_{pol}$ state may be a complex where the DNA is bound at the polymerase active site or in a conformation where the primer terminus is properly annealed.

because Mg^{2+} is required for catalysis and may also be required for proper binding and orientation of the substrate (Hsieh *et al.*, 1993). A T4 pol·DNA complex formed in the absence of Mg^{2+} may not be biologically relevant. Further work is currently underway to characterize these complexes.

T4-Initiated Excision of AP from Primer/Templates. For some primer/templates, a biphasic increase in fluorescence was observed for reactions initiated by the addition of T4 pol to DNA. Rates for the rapid phases were similar regardless of DNA sequence. The amplitudes for both phases were sequence dependent, as were the rates for the slow phases. The influences of local DNA sequence on the hydrolytic removal of dAPMP by T4 pol are consistent with the simple model shown in Figure 8. On mixing, T4 pol binds DNA in one of two states: a state which is active for hydrolysis of the terminal nucleotide, $(E\cdot D)_{exo}$, or one that is inactive for hydrolysis, $(E\cdot D)_{pol}$. The active state $(E\cdot D)_{exo}$ reacts quickly to give a small burst of formation of dAPMP product, which gives rise to the rapid phase in reaction time courses. The inactive state $(E\cdot D)_{pol}$ then converts slowly to the active state $(E\cdot D)_{exo}$. Once in the active state, DNA is rapidly hydrolyzed. This slow conversion to the active state is the rate-determining step along this pathway and gives rise to the slow sequence-dependent phase. The populations of these two states, $(E\cdot D)_{pol}$ and $(E\cdot D)_{exo}$ (reflected in the amplitudes of each phase), are DNA sequence dependent. Local DNA sequences that would be predicted to stabilize the region of DNA around the primer terminus lead to a larger amplitude for the slow phase resulting from a larger population of $(E\cdot D)_{pol}$. At the extreme, for some correct pairs, reaction kinetics were monophasic, indicating that very little if any of the $(E\cdot D)_{exo}$ state was populated. Sequences that are predicted to be more stable also show decreased rates for the slow phase, which is postulated to be the conversion to the active, $(E\cdot D)_{exo}$, state. A decrease in amplitude of the fast phase is correlated with a decrease in rate of the slow phase. Thus, as the overall rate of conversion of $(E\cdot D)_{pol}$ to $(E\cdot D)_{exo}$ decreases, the relative population of the $(E\cdot D)_{exo}$ state decreases.

Our interpretation of the pre-steady-state excision of AP, as measured by a biphasic increase in fluorescence, is consistent with a stopped-flow analysis of the exonuclease associated with T7 DNA polymerase (Donlin *et al.*, 1991) but differs, in one respect, from that proposed for T4 pol (Capson *et al.*, 1992). In the previously proposed kinetic scheme for excision by T4 pol, direct access to the $(E\cdot D)_{exo}$ state from solution was not present; i.e., the conversion of $E + D \rightleftharpoons (E\cdot D)_{exo}$ did not occur (see Figure 8). Binding of T4 pol to DNA resulted in formation of the inactive $(E\cdot D)_{pol}$ complex, which was then able to convert the $(E\cdot D)_{exo}$ complex. Direct access to the $(E\cdot D)_{exo}$ state was not believed to occur because T4-initiated kinetics were monophasic.

For some DNA sequences, we observed biphasic kinetics in T4 pol-initiated reactions which suggest that direct access to the $(E\cdot D)_{exo}$ state is possible. Perhaps the active $(E\cdot D)_{exo}$ state is a polymerase·DNA complex where the DNA is bound at the exonuclease active site and the inactive $(E\cdot D)_{pol}$ state is a complex where the DNA is bound at the polymerase active site, as for T7 DNA polymerase (Donlin *et al.*, 1991). It is also possible that the $(E\cdot D)_{exo}$ state represents a complex where the primer terminus is separated from the template and accessible to the exonuclease, while the $(E\cdot D)_{pol}$ state represents a complex where the primer terminus is properly annealed. This model is consistent with the data thus far, although the complete kinetic mechanism is likely to be more complex. Further experiments need to be done to determine what the identities of these two states are and whether there is evidence for other intermediate states.

Since excision kinetics appear to correlate with DNA melting temperatures, local DNA sequence may be contributing to the efficiency of proofreading by T4 pol as illustrated in Figure 7. The DNA primer/template may exist in two states: one where the primer/terminus is present as an annealed pair (A) and one where one or more nucleotides at the primer terminus are not properly annealed, i.e., a melted-out state (M) (Clayton *et al.*, 1979). These two states exist in an equilibrium which may be different at an enzyme active site from that in aqueous solution. The selectivity of the exonuclease may rely on the fact that it recognizes a melted-out primer terminus (M) as an active substrate and not that it recognizes the difference between a correct pair and a mispair. Conversely, only a properly annealed primer terminus (A) would be an active substrate for polymerization. Both correctly paired and mispaired primer termini could exist in either the M or the A state. The relative populations of the two states would be determined by their relative base-pairing (H-bonding and base stacking) stabilities, where state A is favored for correct pairs and state M is favored for mispairs. However, an incorrectly paired primer terminus, located in a more stable sequence context, might have a higher probability of being in an annealed (A) state than would a correctly paired terminus located in a less stable sequence context. A striking example of this point is the demonstration that an AP·C mispair adjacent to G·C neighboring base pairs was excised 4-fold more *slowly* than a correct AP·T base pair having A·T neighbors.

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