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Kinetic Partitioning between the Exonuclease and Polymerase Sites in DNA Error Correction†

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ABSTRACT: We present a kinetic partitioning mechanism by which the highly efficient 3′ → 5′ exonuclease activity of T7 DNA polymerase maximizes its contribution to replication fidelity with minimal excision of correctly base-paired DNA. The elementary rate constants for the proposed mechanism have been measured directly from single-turnover experiments by using rapid chemical quench–flow techniques. The exonuclease activity of T7 DNA polymerase toward single-stranded DNA is quite fast ($k_x > 700\ s^{-1}$). This rapid exonuclease is restrained with double-stranded DNA by a kinetic partitioning mechanism that favors the binding of the DNA to the polymerase site to prevent the rapid degradation of matched DNA and yet allows selective removal of mismatched DNAs. Both matched and mismatched DNAs bind tightly to the polymerase site, with approximately equal affinities, $K_d^p = 20$ and 10 nM, respectively. Selective removal of the mismatch is governed by the rate of transfer of the DNA from the polymerase to the exonuclease site ($k_{p\rightarrow x}$). The rapid excision of matched DNA is limited by a slow transfer rate ($k_{p\rightarrow x} = 0.2\ s^{-1}$) from the polymerase to the exonuclease site relative to the rate of polymerization [$k_p = 300\ s^{-1}$; Patel et al. (1991) *Biochemistry* (first of three papers in this issue)]. Removal of mismatched DNA is facilitated by its faster transfer rate ($k_{p\rightarrow x} = 2.3\ s^{-1}$) to the exonuclease site relative to the slow rate of polymerization over a mismatch [$k_p^i = 0.012\ s^{-1}$; Wong et al. (1991) *Biochemistry* (second of three papers in this issue)]. The dissociation rate of the mismatched DNA is slower ($k_{off} = 0.4\ s^{-1}$) than the observed transfer rate ($2.3\ s^{-1}$); thus, an intramolecular transfer of mismatched DNA from the polymerase to the exonuclease site was observed. Bidirectional transfer was observed as the matched DNA generated by exonucleolytic hydrolysis of the mismatched primer/terminus was rapidly transferred from the exonuclease site to the polymerase site ($k_{x\rightarrow p} > 700\ s^{-1}$). Approximately 50% of the mismatched DNA that dissociates rather than transferring intramolecularly to the exonuclease site is also rapidly excised because it binds more rapidly to the exonuclease site than to the polymerase site ($k_{on}^x = 5 \times 10^8\ M^{-1}\ s^{-1}$; $k_{on}^p = 4 \times 10^7\ M^{-1}\ s^{-1}$). The slow polymerization rate onto a mismatch provides time during which an error can be corrected by the kinetic partitioning mechanism. The contribution of the exonuclease activity to fidelity, represented by ϕ_x, can be calculated as

$$\phi_x = \frac{\theta k_{off} + k_{p\rightarrow x} + k_p^i}{k_p^i} = \frac{0.5(0.4) + 2.3 + 0.012}{0.012} = 210$$

where k_{off} is the dissociation rate of the DNA from the polymerase site into free solution, $k_{p\rightarrow x}$ is the transfer rate of the DNA from the polymerase site to the exonuclease site, k_p is the polymerization rate onto a correctly base-paired DNA, k_p^i is the polymerization rate onto a mismatch, and θ is the fraction of DNA excised rapidly upon rebinding. Under our conditions, the cost of this proofreading is minimal, with the loss of only a small fraction of correctly base-paired products, 0.0008.

Most prokaryotic and bacteriophage DNA polymerases have an associated 3′ → 5′ exonuclease activity that hydrolyzes the 3′-base of a DNA chain to dNMP,[1] leaving the DNA strand shortened by one nucleotide:

$$E{\cdot}D_n + H_2O \rightarrow E{\cdot}D_{n-1} + dNMP$$

This exonuclease activity can be found associated with either a distinct domain on the same polypeptide as the polymerase, (e.g., Pol I; Jovin et al., 1969) or on a separate polypeptide in a multienzyme complex (e.g., the ε subunit of Pol III; Maki & Kornberg, 1987). Brutlag and Kornberg (1972) first demonstrated with Pol I that a 3′ terminal mismatch is excised before the DNA can be elongated. The importance of the exonuclease activity in vivo has been established by several studies. DiFrancesco et al. (1984) directly correlated an observed increase in the in vivo mutation frequency with a reduction in the 3′ → 5′ exonuclease activity of Pol III. Isolation and characterization of mutant bacteriophage T4 DNA polymerases with altered ratios of exonuclease to polymerase activity have revealed an inverse relationship between this ratio and the in vitro error frequency (Muzyczka et al., 1972; Reha-Krantz & Bessman, 1977). Furthermore, Hershfield and Nossal (1972) have demonstrated that the exonuclease activity of T4 DNA polymerase preferentially removes unpaired 3′-termini. Altogether, these findings indicate that the 3′ → 5′ exonuclease activity is responsible for correction of

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[1] Abbreviations: dNMP, deoxynucleoside 5′-monophosphate; dNDP, deoxynucleoside 5′-diphosphate; dNTP, deoxynucleoside 5′-triphosphate; dATP, deoxyadenosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; Pol I, *Escherichia coli* DNA polymerase I; Pol III, *E. coli* DNA polymerase III.

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errors made by the polymerase. However, due to lack of kinetic data on the exonuclease activity, the basis of the selective exonucleolytic repair cannot be directly established or quantified. For example, it has not been established whether proofreading selectivity is due to a faster rate of excision of a mismatch or due to a slower rate of polymerization over a mismatch, or a combination of both.

Much of our structural understanding of DNA polymerases and their exonuclease activities are derived from the crystal structure of Klenow fragment of Pol I. Based on this information, a model has been proposed in which the duplex DNA containing the mismatch must first be melted for the resulting single-stranded region to move into an adjacent exonuclease site before excision (Ollis et al., 1985). Since the two sites in the crystal structure are separated by approximately 30 Å, four to five bases must melt in order to span that distance in Klenow. However, little information is available to describe the kinetics and thermodynamics that govern the movement of the DNA between the polymerase and exonuclease sites.

The data of Kuchta et al. (1988) for Klenow suggest an insignificant contribution of its exonuclease activity to fidelity, if the calculation is done properly by kinetic partitioning including the rapid release of the DNA from the enzyme. For Klenow, the most likely event following a misincorporation is the dissociation of the DNA from the enzyme (greater than 95% probability) (Kuchta et al., 1988; Joyce, 1989). Nonetheless, in order to explain the failure to observe the accumulation of the products of misincorporation in solution, an additional error correction step was postulated to occur prior to dissociation of the enzyme–DNA complex (Kuchta et al., 1988). However, no direct evidence was provided for this hypothetical editing step, and other explanations of the data are possible. Thus, the small, nearly negligible contribution of the exonuclease towards fidelity of Pol I may reflect its physiological role as a repair enzyme. Therefore, we reason that the high exonuclease activity of T7 DNA polymerase would make this enzyme a more suitable model for studying the role of exonucleolytic proofreading in DNA replication fidelity.

The DNA polymerase of bacteriophage T7 is a heterodimer consisting of the phage encoded gene 5 protein and the *Escherichia coli* encoded thioredoxin protein (Mark & Richardson, 1976). The 3′ → 5′ exonuclease activity of T7 DNA polymerase resides wholly within the gene 5 protein. Thioredoxin functions as an accessory protein in the complex (Adler & Modrich, 1979; Hori et al. 1979). In the previous two papers of this series, we have established a model for fidelity of DNA polymerization based on a detailed kinetic analysis of correct and incorrect incorporation (Patel et al., 1991; Wong et al., 1991). This paper describes the contribution of the 3′ → 5′ exonuclease activity to fidelity and provides the first complete description of kinetic partitioning of DNA between the exonuclease and polymerase sites.

We have identified and measured the individual steps in this kinetic pathway for exonucleolytic proofreading by using defined oligonucleotide primer/templates and rapid chemical quench–flow techniques. The kinetic partitioning of the mismatched DNA to the exonuclease site and the rapid, selective removal of the mismatched DNA have allowed us to calculate a selectivity parameter, which describes contribution of the exonuclease proofreading to fidelity of DNA replication.

Experimental Procedures

Materials

Enzymes. Phage T7 gene 5 protein was purified from *E. coli* A179/pGP5-3/pGP1 by the procedure of Patel et al. (1991), with one exception. Following the DNA–cellulose column, the protein was loaded onto a hydroxyapatite column (20 cm × 2.5 cm, 100-mL bed volume) and washed with 100 mL of buffer containing 20 mM KPO_4, 2 mM sodium citrate, 0.1 mM DTT, and 10% glycerol. The protein was eluted with a 500-mL linear gradient from 25 to 150 mM KPO_4, with the protein eluting between 60 and 80 mM KPO_4. Fractions containing protein were collected, concentrated by dialysis against buffer containing 50% glycerol [in 40 mM Tris (pH = 7.5), 1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, and 50 mM NaCl], and stored at −80 °C. *E. coli* A179 [Hfr-C-(λ)*trxA*::*kan*] and plasmids pGP5-3 and pGP1 were obtained from S. Tabor and C. C. Richardson (Harvard Medical School). Thioredoxin was purified from *E. coli* SK3981/pBJK8 (provided by B. Schuster, The Pennsylvania State University) by a published procedure (Lunn et al., 1984). Purified Klenow fragment was provided by R. Kuchta and C. Catalano (The Pennsylvania State University). T4 polynucleotide kinase was purchased from New England Biolabs. Unless otherwise specified, the T7 DNA polymerase described in this paper is a reconstituted complex of wild-type T7 gene 5 protein and *E. coli* thioredoxin in a molar ratio of 1:20.

Chemicals. The [α-^{32}P]dNTPs and [γ-^{32}P]ATP were purchased from New England Nuclear or ICN, and their purity was estimated by using PEI-cellulose thin-layer chromatography plates (EM Science) developed in 0.3 M potassium phosphate (pH = 7.0) to resolve the dNTP, dNDP, and dNMP species. All nonradioactive dNTPs were purchased from Pharmacia. DE81 filters (2.5 cm) were from Whatman. Heparin (grade II) was purchased from Sigma. Hydroxyapatite resin was Spectra-gel HAP from Spectrum.

Synthetic Oligonucleotides. The oligonucleotides (Table I) were synthesized on either an Applied Biosystems 380A DNA synthesizer or a Milligen/Biosearch 7500 DNA synthesizer and purified by electrophoresis through a denaturing gel (18% acrylamide, 1.5% bisacrylamide, and 8 M urea in TBE buffer). The major DNA band was visualized through UV shadowing, excised, and electroeluted from the gel slice in an Elutrap apparatus (Schleicher & Schuell). Concentrations of the purified oligonucleotides were determined by UV absorbance at 260 nm with the following extinction coefficients: 25-mer, ϵ = 249 040; 26T-mer, ϵ = 265 840; $26T_2$-mer, ϵ = 274 240; $26T_3$-mer, ϵ = 282 640; 36-mer, ϵ = 377 000 cm^2/μmol. The extinction coefficients were calculated from the sum of extinction coefficients of all bases in the oligonucleotide. Duplex oligonucleotides were annealed at room temperature in TE buffer and then purified by electrophoresis through a nondenaturing gel (20% acrylamide and 1.5% bisacrylamide in TBE buffer). The major DNA band was visualized, excised, and electroeluted as described above.

Table I: Oligonucleotides

25/36-mer	5′-GCCTCGCAGCCGTCCAACCAACTCA CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT-5′
26T/36-mer	5′-GCCTCGCAGCCGTCCAACCAACTCAT^{T} CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT-5′
$26T_2$/36-mer	5′-GCCTCGCAGCCGTCCAACCAACTCAT^{TT} CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT-5′
$26T_3$/36-mer	5′-GCCTCGCAGCCGTCCAACCAACTCAT^{TTT} CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT-5′

Buffers. All experiments with T7 DNA polymerase were carried out in buffer containing 40 mM Tris (pH = 7.5), 1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, and 50 mM NaCl. This buffer with magnesium also contained 12.5 mM

$MgCl_2$. Other buffers were used for purifications: TBE buffer is 90 mM Tris (pH = 7.5), 90 mM boric acid, and 2 mM EDTA, and TE buffer is 10 mM Tris (pH = 7.5) and 1 mM EDTA. All experiments were performed at 20 °C.

Methods

5′ ^{32}P Labeling of Oligonucleotides. Single-stranded 29-mer was 5′-radiolabeled with T4 polynucleotide kinase as described by Maniatis et al. (1982) for protruding 5′-termini. Duplex 25/36-mer, 26T/36-mer, $26T_2$/36-mer, and $26T_3$/36-mer were 5′-labeled under the same conditions, with one modification: the protruding 5′-terminus of the template strand was incubated with an equimolar amount of cold ATP at room temperature for 10 min prior to addition of [γ-^{32}P]ATP. This allowed for even labeling of the two 5′-termini. Unincorporated nucleotides were removed by using Bio-Spin-30 columns (Bio-Rad Laboratories).

3′ ^{32}P Labeling of 25/36-mer. Primer/template 25/36-mer was labeled at the 3′-terminus with [α-^{32}P]dATP by using 1 μM Klenow fragment. The reaction was incubated at room temperature for 30 min and quenched with EDTA at a final concentration of 0.1 M. The solution was extracted once with an equal volume of phenol–chloroform (1:1), and the unincorporated nucleotides were removed with a Bio-Spin-30 column.

Concentration of 3′-Mismatched 26T/36-mer. The concentration of 3′-mismatched 26T/36-mer was determined by excision of the mismatched T in the 27th position and subsequent incorporation of two [α-^{32}P]dCTPs with T4 DNA polymerase (purified by I. Wong in this lab). The 26T/36-mer (200 nM) and dCTP (20 μM) were incubated at room temperature for 1 min. T4 DNA polymerase (10 nM) and Mg^{2+} (12.5 mM) were added to initiate the reaction; the reaction was quenched with EDTA to a final concentration of 0.25 M at time intervals from 5 s to 10 min. Incorporation of [α^{32}-P]dCTP was monitored by a DE81 filter binding assay (Bryant et al., 1983) and quantitated by liquid scintillation counting.

Reconstitution of T7 DNA Polymerase. T7 DNA polymerase was reconstituted immediately prior to use. Thioredoxin was added to the standard buffer (40 mM Tris (pH = 7.5), 1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, and 50 mM NaCl), the solution was incubated at 4 °C for 2–3 min, and then wild-type gene 5 protein was added to obtain a final molar ratio of 1:20 (gene 5 protein to thioredoxin).

Rapid-Quench Experiments. Rapid-quench experiments were performed by using an apparatus designed and built by K. A. Johnson (1986). The reactions were carried out by two different methods. First, because of the absolute requirement of Mg^{2+} for T7 DNA polymerase enzymatic activity, the enzyme and DNA could be preincubated together in buffer without Mg^{2+} and the reaction was initiated by addition of Mg^{2+}. Alternatively, the enzyme and DNA could be incubated separately in the presence of Mg^{2+} and the reaction was initiated by mixing the enzyme and DNA. In all cases, concentrations of enzyme and substrates cited in the text are those after mixing (during the enzymatic reaction). Reactions were quenched with 0.3 M EDTA after time intervals of 3 ms–20 s. An EDTA concentration of 0.3 M was sufficient to completely stop the reaction (Patel et al., 1991).

Product Analysis. Reaction products from 5′-labeled substrates were analyzed by electrophoresis through denaturing sequencing gels and were quantified by excising and counting the radioactivity in the bands in a Beckman LS7000 scintillation counter. Reaction products from 3′-labeled substrates were quantitated by following the loss of the 3′-radiolabel by using a DE81 filter binding assay (Bryant et al., 1983).

Data Analysis. The kinetic data were modeled by using the KINSIM kinetic simulation program, kindly provided by Carl Frieden and Bruce Barshop (Washington University, St. Louis, MO; Barshop et al., 1983) and refined by regression analysis to obtain the best fit by using a modification of the program of Zimmerlie and Frieden (1989). The KINSIM program was run on a Digital Microvax II computer. The data were fit to the mechanism shown in Scheme I; the rate constants are shown in Table II. Data analysis, when fitting to a single mathematical expression by nonlinear regression, was performed by using the program RS/1 (BBN Software Products Corp., Cambridge, MA), also run on a Digital Microvax II computer.

Scheme I

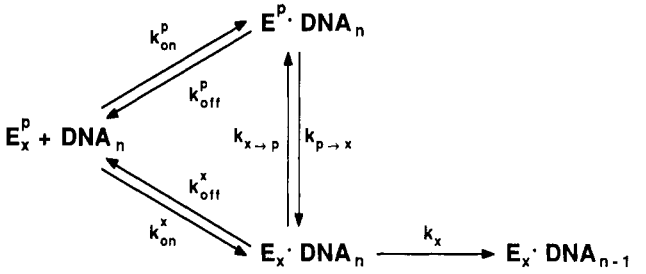


Table II: Kinetic Constants for 26T/36-mer

K^x_d [a]	1.2–2.8 μM	k^p_{off} [b]	0.40 s^{-1}
K^p_d [b]	9 nM	k_x [a]	896 ± 14 s^{-1}
k^x_{on} [a]	(4–6) × 10^8 M^{-1} s^{-1}	$k_{p\to x}$ [b]	2.3 s^{-1}
k^p_{on} [b]	4 × 10^7 M^{-1} s^{-1}	$k_{x\to p}$ [a]	714 ± 14 s^{-1}
k^x_{off} [a]	1022 ± 43 s^{-1}		

[a] These kinetic constants were determined by computer simulation of the data by using the mechanism given in Scheme I. The limits listed are those as constrained by the mechanism and necessary to fit the data by computer simulation. Conditions: 40 mM Tris (pH = 7.5), 12.5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 0.1 mg/mL BSA at 20 °C. [b] These kinetic constants were measured directly from pre-steady-state experiments and the error determined by RS1 in fitting to a single mathematical expression.

RESULTS

Exonuclease Activity of T7 DNA Polymerase on a Single-Stranded DNA. The rate constant for hydrolyzing the 3′-base of a single-stranded DNA substrate was estimated from a pre-steady-state kinetic experiment. This experiment was carried out by using a rapid chemical quench–flow instrument because of the fast exonucleolytic rate of T7 DNA polymerase. The excision rate on single-stranded DNA was measured by mixing a solution of T7 DNA polymerase (2.0 μM) with a single-stranded 29-mer (100 nM) and Mg^{2+} to initiate the reaction, which then was quenched with 0.3 M EDTA at time intervals ranging from 3 to 600 ms. Pre-steady-state conditions were ensured by using an excess concentration of the polymerase over the DNA. The resulting time course (Figure 1) shows that T7 DNA polymerase hydrolyzed single-stranded DNA rapidly; the reaction was essentially complete in 3 ms, which implied a rate in excess of 700 s^{-1}. By computer simulation, the loss of starting material and the formation of consecutive excision products could be fit to obtain a better estimate of the excistion rate constant of 800 ± 200 s^{-1}. The data were also fit to provide an estimate of the rates of binding and dissociation of the enzyme–DNA complex, k_{on} = (4–6) × 10^8 M^{-1} s^{-1} and k_{off} = 900 ± 200 s^{-1}. It was necessary to make the value for k_{on} for the binding of DNA to the exonuclease site high in order to account for the full burst am-

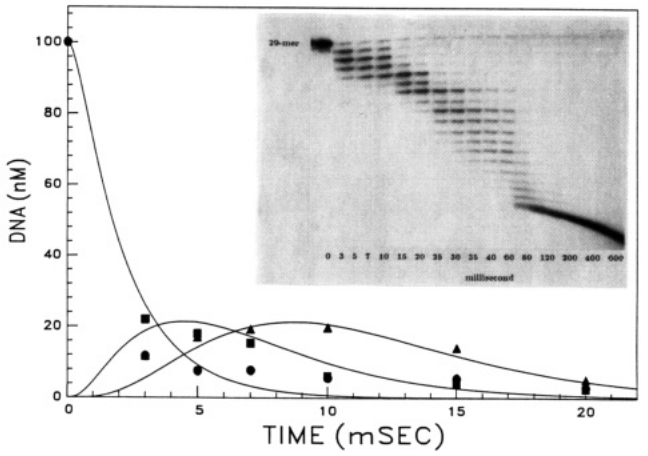


FIGURE 1: Exonuclease activity on single-stranded DNA. The single-stranded exonuclease reaction was carried out with T7 DNA polymerase (2 μM) and 5′-radiolabeled single-stranded 29-mer (100 nM) incubated in the presence of Mg^{2+} (12.5 mM) in separate syringes of a rapid-quench apparatus. The reaction was initiated by mixing enzyme with DNA and quenched with EDTA (0.3 M) after time intervals from 3 to 600 ms. The reaction products were analyzed on a denaturing sequencing gel. Shown in the inset is the autoradiograph from the excision reaction on single-stranded 29-mer. Computer simulation of the loss of the 29-mer (●) and generation of the exonuclease products 27-mer (■) and 25-mer (▲) allowed estimation of k_{on} as (4–6) × 10^8 M^{-1} s^{-1}; k_{off} was estimated to be from 700 to 1100 s^{-1}, and k_x was estimated to be from 600 to 1000 s^{-1}.

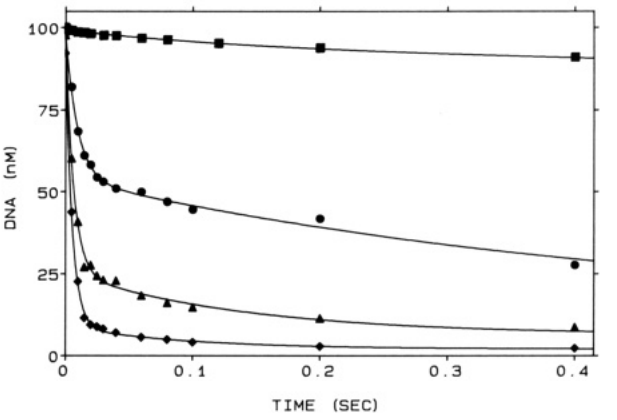


FIGURE 2: Comparison of exonuclease activity on duplex oligonucleotides containing zero to three 3′-mismatches. T7 DNA polymerase (300 nM) and 5′-labeled primer–templates (100 nM) were incubated in the presence of Mg^{2+} in separate syringes of a rapid-quench apparatus, and the reaction was initiated by mixing enzyme with DNA. The reaction was quenched with EDTA (0.3 M) after time intervals from 5 to 400 ms, and the excision products were analyzed on denaturing sequencing gels. The excision rate for the loss of the 3′-base was determined by fit to a double-exponential curve with the following results: The matched substrate, 25/36-mer (■), fit to a rate of 7.4 s^{-1} for 5% of the DNA, and the second exponential fit to a rate of 0.16 s^{-1} for 61% of the DNA. The single mismatch, 26T/36-mer (●), fit to a rate of 105 s^{-1} for 46% of the DNA; the double mismatch, $26T_2$/36-mer (▲), fit to a rate of 150 s^{-1} for 70% of the DNA; and the triple mismatch, $26T_3$/36-mer (◆), fit to a rate of 185 s^{-1} for 85% of the DNA.

plitude of hydrolysis observed in the time course. These values for k_{on} and k_{off} translated into a K_d at the exonuclease site of 2 ± 0.8 μM.

In contrast, the 3′-base of a duplex DNA was excised at a relatively slow rate of 0.16 s^{-1} (Figure 2). This large difference between the excision rates of duplex and single-stranded DNA clearly demonstrated that a single-stranded DNA was the preferred substrate for the 3′ → 5′ exonuclease activity of T7 DNA polymerase. This result suggested that the single-stranded character of the primer/terminus should greatly affect the rate and extent of the exonuclease activity.

Exonuclease Activity on DNAs with Zero to Three 3′-Mismatches. Pre-steady-state kinetic experiments to measure the excision rates with various primer/templates containing zero to three 3′-mismatches (25/36-mer, 26T/36-mer, $26T_2$/36-mer, and $26T_3$/36-mer; Table I) were carried out to further investigate the mechanism of the 3′ → 5′ exonuclease reaction. The single-turnover excision rates of the 3′-base for all the primer/templates were measured by mixing a solution containing DNA (100 nM) and Mg^{2+} with T7 DNA polymerase (300 nM) to initiate the exonuclease reactions. The time course of the exonuclease reaction on each substrate is shown in Figure 2. For each of the substrates, the exonuclease time course was biphasic; both the rate and the amplitude of the fast phase increased with increasing number of 3′-mismatches. For the matched substrate, 25/36-mer, the rate of the fast phase was 7.5 ± 0.5 s^{-1} with an amplitude equal to 5% of the DNA. The second exponential was fit to a rate of 0.16 ± 0.01 s^{-1}. For one mismatch, 26T/36-mer, the fast phase proceeded at a rate of 105 ± 10 s^{-1} with an amplitude of 46% of the DNA; for $26T_2$/36-mer, the fast phase was 150 ± 12 s^{-1} for 70% of the DNA; and for the $26T_3$/36-mer, it was 185 ± 15 s^{-1} for 85% of the DNA. It is important to note that, in each case, the time dependence of the reaction describes the removal of the first 3′-terminal nucleotide, and the reaction was carried out with enzyme in excess over the DNA. Thus the biphasic kinetics reflect events occurring within a single turnover of the exonuclease reaction.

If the exonuclease rate for a single-stranded DNA corresponds to the maximum rate at which the 3′-base of a single-stranded DNA would be excised, then one might expect the rate of the fast phase to be >700 s^{-1} (the single-stranded excision rate). The slower rates observed under the given experimental conditions are attributed to a rate-limiting binding of the DNA to the enzyme. This interpretation was confirmed by repeating the experiment at different concentrations of enzyme, giving a rate proportional to enzyme concentration (data not shown). This association rate was calculated by fitting the data by computer simulation for a single-mismatched substrate, 26T/36-mer, and assuming an excision rate of 700 s^{-1} to give a value of 5 × 10^8 M^{-1} s^{-1}; this value is within the range given for association of single-stranded DNA at the exonuclease site (Figure 1). Therefore, an increase in rate with the number of mismatches was partly due to a corresponding increase in the rate of binding to the exonuclease site. Keeping in mind that the experiment was performed with enzyme in excess over substrate, one must address the question as to why excision of the 3′-base of each substrate did not go to completion and why the amplitude of the fast phase increased with the increasing number of 3′-mismatches. The result from the next experiment provided an explanation.

DNA Binding Preference for the Polymerase versus the Exonuclease Site. If the burst amplitude and rate observed in the above experiment are limited by the rate of binding of the DNA to the enzyme, then upon preincubation of the DNA with the enzyme we might observe an exonuclease rate of >700 s^{-1}. Accordingly, DNA polymerase (300 nM) was preincubated with 26T/36-mer (100 nM) in the absence of Mg^{2+}, and the exonuclease reaction was started by the addition of buffer containing Mg^{2+}. The resulting time course of the exonuclease reaction is shown in Figure 3. Instead of observing a faster reaction when the DNA was preincubated with the enzyme, the rate and amplitude of the fast phase were decreased and we observed only a slow excision rate. The data fit a single

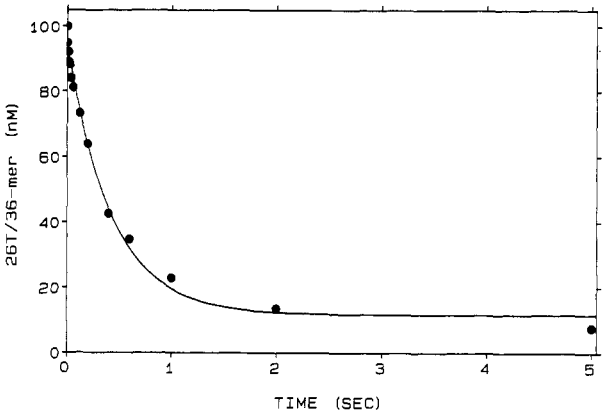


FIGURE 3: Determination of rate of switch between polymerase and exonuclease sites for 26T/37-mer. In one syringe of a rapid-quench apparatus, T7 DNA polymerase (300 nM) and 5′-radiolabeled 26T/36-mer (100 nM) were preincubated in the absence of Mg^{2+}, and the reaction was initiated with the addition of Mg^{2+} (12.5 mM) (●). The reaction was quenched with EDTA (0.3 M) after time intervals from 5 ms to 5 s, and the excision products were analyzed on a denaturing sequencing gel. The data for loss of the 3′-mismatch were fit to a single-exponential curve with a rate 2.3 ± 0.2 s^{-1}.

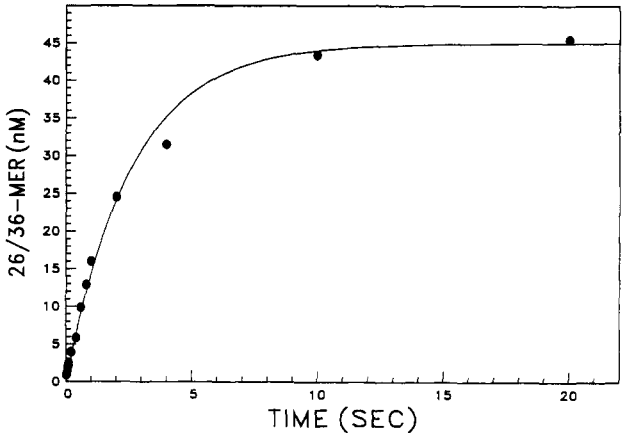


FIGURE 4: Determination of k_{off} for 26T/36-mer from the polymerase site. In one syringe of a rapid-quench apparatus, 5′-labeled 26T/36-mer (40 nM) was incubated with exo⁻ T7 DNA polymerase (400 nM) in the presence of Mg^{2+} (12.5 mM), and the reaction was initiated with the addition of wild-type T7 DNA polymerase (1 μM). The reaction was quenched at time intervals from 5 ms to 20 s with EDTA (0.3 M), and the excision products were monitored on a denaturing sequential gel. The data for generation of excision products (●) were fit to a single exponential with a dissociation rate of 0.40 ± 0.04 s^{-1}.

exponential with a rate of 2.3 ± 0.2 s^{-1}, which was equal to the rate of the slow phase of the kinetics when the reaction was initiated by mixing 26T/36-mer with the enzyme. This result can be explained if the DNA in an equilibrated E·DNA complex was bound at the polymerase site and transferred only slowly to the exonuclease site. In support of this assertion, other experiments have demonstrated that a preincubated E·DNA complex can be elongated rapidly and without a lag (Patel et al., 1991). We propose that, when given time to equilibrate with the enzyme, the DNA binds preferentially to the polymerase site and the observed rate under these conditions (2.3 s^{-1}) is limited by the rate of transfer between the sites. The faster excision rate observed when the reaction was started by mixing enzyme and DNA can be explained if the DNA binds to the exonuclease site faster than it binds to the polymerase site. The amplitude of the fast phase is a function of the kinetic partitioning between rates of excision and sliding of the DNA from the exonuclease site into the polymerase site (see Scheme I).

There are two possible pathways by which a mismatched DNA can transfer from the polymerase to the exonuclease site. The DNA can either dissociate from the polymerase site into free solution and then rebind to the exonuclease site (intermolecular transfer) or the DNA can directly channel into the exonuclease site without dissociation (intramolecular transfer). The slow exonuclease rate constant of 2.3 s^{-1} observed for a preincubated 26T/36-mer could correspond either to dissociation of the DNA from the polymerase site or to direct transfer of the DNA from the polymerase to the exonuclease site. To resolve this, the following experiment was designed to measure directly the rate of dissociation of the DNA from the polymerase site.

Dissociation Rate of a Single-Mismatched Primer/Template (26T/36-mer) from the Polymerase Site. The rate constant for dissociation (k_{off}) of 26T/36-mer from the polymerase site was measured in the following manner. DNA (26T/36-mer; 40 nM) was preincubated with an excess of a 3′ → 5′ exonuclease deficient (exo⁻) mutant (Patel et al., 1991) of T7 DNA polymerase (400 nM) in the absence of Mg^{2+}. The exonuclease reaction was initiated by mixing the complexed DNA with a large excess of wild-type T7 DNA polymerase (1 μM) and Mg^{2+}. Under these conditions, the measured exonuclease rate is limited by the dissociation of the DNA from the polymerase site, because the subsequent binding of the 26T/36-mer and excision of the mismatch by the wild-type enzyme are fast. Figure 4 shows the resulting time course of 26T/36-mer excision. The kinetics fit a single exponential with a rate of 0.4 ± 0.04 s^{-1}, which defines the dissociation rate constant of 26T/36-mer from the polymerase site of the exo⁻ enzyme. Since the rate constants for the polymerase activity of the exo⁻ T7 DNA polymerase are essentially unchanged from those of the wild-type enzyme (Wong et al., 1991), the dissociation rate of 26T/36-mer from the polymerase site of the wild-type enzyme would also be 0.4 s^{-1}.

The excision rate (2.3 s^{-1}) of 26T/36-mer under conditions of preincubation of enzyme and DNA was 6 times faster than the measured dissociation rate constant of 0.4 s^{-1}; therefore, this excision rate could correspond only to the intramolecular transfer rate of the DNA from the polymerase into the exonuclease site. This was confirmed by the following trap experiment. An exonuclease time course was measured by reacting a preincubated T7 DNA polymerase–26T/36-mer complex (300 nM: 100 nM) with Mg^{2+} in the presence of heparin (2 mg/mL), which serves efficiently to trap free enzyme. An excision rate of 2.3 s^{-1} was also observed under these conditions, supporting the proposed intramolecular transfer of the 26T/36-mer from the polymerase to the exonuclease site (data not shown).

K_d and k_{on} of a Single-Mismatched DNA (26T/36-mer) at the Polymerase Site. The equilibrium dissociation constant of 26T/36-mer ($K_d^{26T/36}$) at the polymerase site was determined by a direct competition experiment. The *apparent* K_d for binding of the 25/36-mer to the polymerase site was measured from the concentration dependence of the rate of the burst of dTTP incorporation by T7 DNA polymerase (100 nM) as described by Patel et al. (1991) but in the presence of a fixed concentration of competing 26T/36-mer (100 nM). The K_d^{app} of the 25/36-mer was measured as 212 ± 56 nM (Figure 5). The K_d of the 26T/36-mer was derived from the relationship

$$K_d^{app} = K_d^{25/36}\,(1 + [D]/[K_d^{26T/36}])$$

where [D] is the concentration of 26T/36-mer during the reaction. Using the values $K_d^{25/26}$ = 18 nM (Patel et al., 1991) and [D] = 100 nM, we calculated $K_d^{26/36}$ to be 9 ± 3 nM.

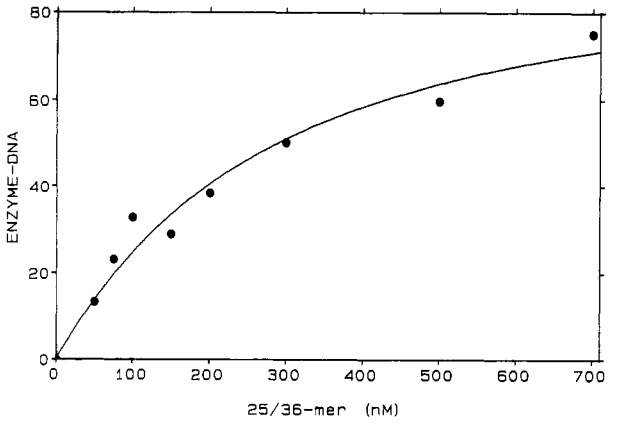


FIGURE 5: Measurement of K_d^{app} of 25/36-mer at the polymerase site. T7 DNA polymerase and 26T/36-mer (100 nm:100 nM) and 25/36-mer varying from 50 to 700 nM were preincubated in the absence of Mg^{2+} in one syringe of a rapid-quench apparatus. The reaction was initiated with the addition of dTTP (200 μM) and Mg^{2+} (12.5 mM) and quenched with EDTA (0.3 M) at 0, 5, 30, and 100 ms. The amount of dTTP incorporation into the 25/36-mer was measured at each concentration, and the amplitude of dTTP incorporation (E·DNA) was plotted as a function of the 25/36-mer concentration. K_d^{app} was determined by fit to the quadratic equation $E{\cdot}DNA = [(K_d^{app} + [D] + [E]) - [(K_d^{app} + [D] + [E])^2 - 4[D][E]^{1/2}]/2$, where [E] = the concentration of T7 DNA polymerase and [D] = the concentration of 26T/36-mer during the reaction. A value of 212 nM was calculated for the 25/36-mer at the polymerase site. Because the true K_d for binding the 25/36-mer at the polymerase site is 18 nM, this measurement allows calculation of K_d = 9 nM for binding of the 26T/36-mer to the polymerase site.

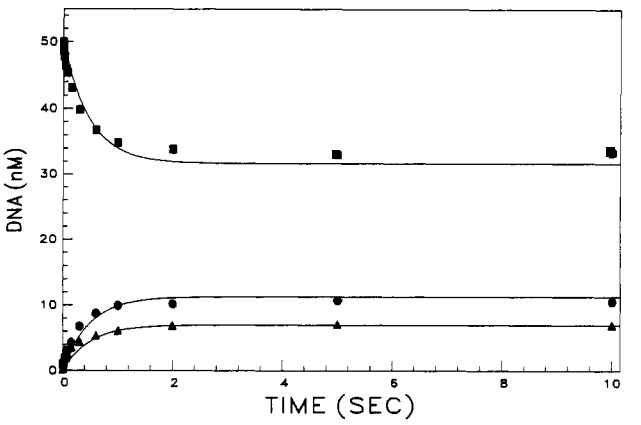


FIGURE 6: Bidirectional channeling of DNA between polymerase and exonuclease sites. T7 DNA polymerase (50 nM) was preincubated with 5′-labeled 26T/36-mer (50 nM) in one syringe of a rapid-quench apparatus, and the reaction was initiated with the addition of Mg^{2+} (25 mM), heparin (2 mg/mL) and dCTP (20 μM). The reaction was quenched after time intervals from 5 ms to 10 s with EDTA (0.3 M). The loss of the 3′-base from the 26T/36-mer (■), the generation of polymerization product, matched 28/36-mer (▲), and the generation of excision products (●) were monitored on a denaturing sequencing gel. The data were fit by computer simulation using the mechanism in Scheme I, the rate constants listed in Table II, and a polymerization rate of 300 s^{-1} (Patel et al., 1990).

By using this value and the equation for K_d, $K_d = k_{off}/k_{on}$, the k_{on} for the mismatched DNA at the polymerase site could be estimated. By using K_d = 9 nM and k_{off} = 0.4 s^{-1}, the k_{on} for 26T/36-mer was calculated to be 4×10^7 M^{-1} s^{-1}. This lower value for k_{on} at the polymerase site relative to k_{on} at the exonuclease site reinforces our interpretation of the exonuclease kinetics. The excision rate is faster when the reaction is initiated by mixing enzyme and DNA rather than by the addition of Mg^{2+} to a preformed enzyme–DNA complex because the DNA binds faster to the exonuclease site but more tightly to the polymerase site. The biphasic kinetics shown in Figure 2 are a function of the initial binding of the DNA to the exonuclease, where a fraction of DNA is excised before it slips into the thermodynamically more favorable polymerase site.

Calculation of Transfer Rate of 26T/36-mer from the Exonuclease Site to the Polymerase Site. The kinetic partitioning model shown in Scheme I was supported by all the single-turnover experiments. The rates were constrained by the thermodynamic box, which requires the ratio of the K_ds for the two sites on the enzyme to be equal to the ratio of the transfer rates between the two sites:

$$\frac{K_d^{p}}{K_d^{x}} = \frac{k_{p\rightarrow x}}{k_{x\rightarrow p}}$$

If K_d^p = 9 nM, $k_{p\rightarrow x}$ = 2.3 s^{-1}, and K_d^x = 1.2–2.8 μM, then $k_{x\rightarrow p}$ = 500–900 s^{-1}. These values were checked for validity in the trapping experiment described below.

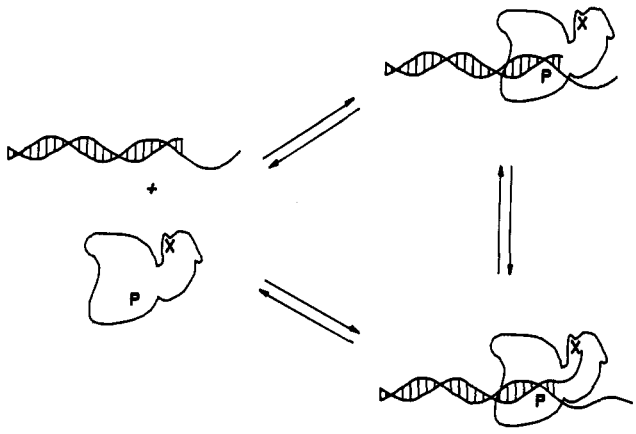
Bidirectional Channeling of DNA between the Polymerase and Exonuclease Sites. To determine whether the DNA is able to channel between the two sites in both directions without dissociation into the free solution, and whether the rate constants obtained previously were valid under conditions of polymerization, a trapping experiment was carried out. We reacted DNA containing a single 3′-terminal mismatch (26T/36-mer) with the dNTP that correctly replaces the mismatched nucleotide (dCTP) in the presence of heparin to trap free enzyme. Since incorporation of the dCTP on top of the mismatch is too slow to observe on the time scale of the experiment (Wong et al., 1991), elongation would occur only if the mismatch was removed first and then dCTP was incorporated in its place. The presence of the heparin trap to sequester free polymerase ensures that the observed exonucleolytic and polymerization reactions occur without dissociation and reassociation of the DNA. The experiment was conducted by preincubating T7 DNA polymerase (50 nM) with 26T/36-mer (50 mM) and initiating the reaction by adding Mg^{2+}, heparin (2 mg/mL), and dCTP (20 μM). Figure 6 shows the time course of loss of the mismatch, accumulation of the excision product, 26/36-mer, and accumulation of the final polymerization product involving the addition of two dCMP residues, 28/36-mer. Because the reaction was carried out in the presence of an efficient trapping agent, both reactions must have occurred without intermediate DNA dissociation, demonstrating that DNA can channel between the two sites.

Evidence for the validity of the previously determined rate constants under the conditions of polymerization was provided by the fact that the kinetic data for loss of the 3′-mismatch and generation of excision and polymerization products were fit by computer simulation using the model in Scheme I and the rate constants in Table II. Simulation of the data from this experiment and the experiment shown in Figure 2, with the 26T/36-mer, allowed refinement and calculation of the error for the values of k_x, k_{off}, and $k_{x\rightarrow p}$ for the 26T/36-mer. By setting the values for k_{on}^x and $k^x{}_{off}$, simulation of Figure 2 gave values of $k_x = 896 \pm 14$ s^{-1} and $k_{x\rightarrow p} = 714 \pm 14$ s^{-1} by regression analysis. Simulation of the data from Figure 6, with the values of k_x and $k_{x\rightarrow p}$ set from the above simulation, gave a value of $k_{off}^x = 1022 \pm 43$ s^{-1}.

Discussion

The fundamental problem facing any exonucleolytic error correction mechanism is the need to optimize accuracy without sacrificing speed. An active but indiscriminate exonuclease would remove mismatches at a high rate, but at the cost of

Scheme II



excessive and unnecessary removal of correctly base-paired DNA. On the other hand, a slower exonuclease might be inefficient in correcting errors, resulting in loss of fidelity. On the basis of our detailed examination of the pre-steady-state kinetics of the exonuclease activity of T7 DNA polymerase, we have provided a quantitative evaluation of the contributions of the various reactions to the simple kinetic partitioning mechanism (illustrated in Scheme II), which effectively accounts for both its efficiency and high selectivity in error correction. The kinetic constants represented by Scheme I and listed in Table II provide a quantitative definition of the reaction governing error correction according to the model shown in Scheme II. This single, unifying model quantitatively accounts for all of our experimental results.

There are three important properties of the kinetic partitioning between the polymerase and exonuclease sites that lead to efficient proofreading: (1) The binding of DNA to the polymerase site is thermodynamically favored over the binding to the exonuclease site; we estimate an equilibrium constant of approximately 100. (2) The DNA is able to channel between the polymerase and exonuclease sites, in both directions, without dissociating from the enzyme. A 10-fold selectivity of the exonuclease for mismatched DNA is due to a faster rate of migration from the polymerase site to the exonuclease site. (3) Overall selectivity is largely attributable to the slower rate of polymerization on top of a mismatch, such that the stalling of the enzyme after a misincorporation allows time for the transfer of the DNA to the exonuclease site; this circumvents the need for a highly active or highly selective exonuclease.

Our data provide an explanation for previous observation on the crystal structure of Klenow and of the higher activity of the 3′ → 5′ exonuclease toward single-stranded DNA (Hershfield & Nossal, 1972), each of which argues that the exonuclease site recognizes only the single-stranded portion of DNA. We have measured directly the rate constant for hydrolysis of single-stranded DNA ($k_x < 1000\ s^{-1}$) and have set that as our limit for the activity toward duplex DNA melted out into the exonuclease site. We have also discovered that duplex DNA binds more rapidly to the exonuclease site with a binding rate constant approaching the diffusion-controlled limit ($>4 \times 10^8\ M^{-1}\ s^{-1}$). The binding to the polymerase site is slower ($<6 \times 10^7\ M^{-1}\ s^{-1}$) but more thermodynamically favored ($K_d \leq 20$ nM). Interestingly, the affinity of the duplex DNA containing a single mismatch for the polymerase is not altered by the presence of a mismatch, at least within the limits of error in making the measurement. A correctly base-paired primer/template binds preferentially in the polymerase site and tends to remain at the polymerase site; in the presence of dNTPs, processive DNA synthesis in the polymerase site takes place at $300\ s^{-1}$ (Patel et al., 1991), which effectively competes with the transfer rate to the exonuclease site ($k_{p\rightarrow x} = 0.2\ s^{-1}$).

When duplex DNA was reacted with the enzyme without preincubation, a small (~5%) burst of rapid excision was observed (Figure 2) that was not present when the DNA had been preincubated with the enzyme. This 5% corresponds to the estimated fraction of frayed 3′ ends in a solution of duplex DNA (calculated from an approximate ΔG of 2 kcal/mol for an average base-pairing). This fast reaction is observed only when enzyme and DNA have not been preincubated and provides direct evidence that binding at the exonuclease site is kinetically faster whereas binding at the polymerase site is thermodynamically preferred. This phenomenon, which was observed with DNAs containing one to three mismatches (Figure 2), is the key to understanding how the enzyme deals with mismatched DNA. For the triple-mismatched $27T_3$/36-mer, the 3′-terminal mismatch was rapidly excised in a single turnover. In this extreme case, where the 3′ ends were highly single-stranded, the partitioning greatly favored rapid binding at the exonuclease site. In the intermediate cases of one and two mismatches, correspondingly smaller fractions underwent rapid excision. The smaller amplitudes are a function of the thermodynamic preference for binding of the DNA to the polymerase site and the kinetic partitioning between hydrolysis and sliding of the DNA to the polymerase site.

These bursts of rapid excision take place upon initial binding of the enzyme and DNA and are observable only during single-turnover experiments. Given sufficient time for equilibration, the DNA will preferentially bind to the polymerase site. Any subsequent excision would then reflect the significantly slower transfer rate of DNA from the polymerase site to the exonuclease site ($2.3\ s^{-1}$ for a single mismatch and $0.2\ s^{-1}$ for a matched DNA). These are the rates observed in the slow phase of our experiments; they equal the rates observed in steady-state measurements of exonuclease activity.

The similar dissociation constants measured for the matched and mismatched DNAs (K_ds of 18 and 9 nM, respectively) reflect the nature of the protein–DNA interactions. On the basis of analysis of the X-ray structure of Klenow (Ollis et al., 1985), the polymerase contacts approximately 8–10 bases of the duplex region of the primer/template. Consequently, the difference of only a single mispair at the primer terminus is not expected to greatly affect the overall free energy of binding. The apparent tighter binding to the mismatched DNA probably reflects the greater error in this measurement.

The selectivity between matched and mismatched DNA comes from the difference in the transfer rates of the two DNAs. The 12-fold difference in the transfer rate corresponds to a $\Delta\Delta G^{\ddagger}$ of −1.5 kcal/mol in favor of transferring the mismatch into the exonuclease site. This number is consistent with the reported value for a single mismatch based on melting data of duplex oligonucleotides (Petruska et al., 1988). This channeling between the two sites is clearly important for error correction. The trap experiment illustrated in Figure 6 establishes the bidirectionality of this intramolecular transfer process by direct measurement.

During polymerization, the enzyme misincorporates with a frequency of $\sim 1 \times 10^{-6}$, and upon misincorporation, the rate of polymerization over the mismatch is only $0.012\ s^{-1}$ (Wong et al., 1991). This effectively stalls the enzyme, allowing sufficient time for the mismatched DNA to channel at $2.3\ s^{-1}$ into the exonuclease site, where its removal is assured by the fast excision rate of $> 700\ s^{-1}$. This excision rate guarantees

efficiency of repair while kinetic partitioning minimizes its cost.

We define the contribution of the exonuclease to fidelity, ϕ_x, as the sum of the frequencies of errors repaired during this "stall" time according to the general equation

$$\phi_x = \frac{\theta k_{off} + k_{p\rightarrow x} + k_p^i}{k_p^i} = \frac{0.5(0.4) + 2.3 + 0.012}{0.012} = 210$$

where k_{off} is the dissociation rate of the DNA from the polymerase site into free solution, $k_{p\rightarrow x}$ is the transfer rate of the DNA from the polymerase site to the exonuclease site, and k_p^i is the rate of polymerization on a mismatch. Stated in terms of the probability of removing a mismatch, 99.5% of all mismatches are removed.

A small fraction of mismatched DNA will dissociate from the enzyme at 0.4 s^{-1}. These molecules will preferentially rebind to the enzyme in the exonuclease site, and under our conditions, approximately half of the terminal mismatches will then be excised; the remainder will reenter the pool of enzyme-bound DNA to partition once again. The term θ is included to account for the fraction of the DNA that is excised rapidly upon rebinding to the enzyme after a dissociation event. That is, in the kinetic partitioning, the dissociation of the DNA from the enzyme does not directly contribute to fidelity, but upon rebinding of the DNA to the enzyme, we know that 50% of the mismatches are rapidly excised, while the remainder of the DNA falls back into the polymerase site. To a first approximation, we can include the contributions of dissociation to fidelity by including the term θk_{off} toward the net rate of the exonuclease in vitro. The value of θ will vary between 0 and 1, depending upon the fate of the DNA after dissociation. If the DNA simply rebinds with no change in the probability of excision, then $\theta = 0$, and dissociation is considered a nonevent. If the DNA is excised with high efficiency after dissociation, then $\theta = 1$, and dissociation is counted as a step contributing to fidelity. The situation in vitro can be addressed rigorously because we know the fate of the DNA following dissociation and rebinding. However, in vivo, it is possible that other proteins such as exonucleases may bind to the free DNA. In any event, the polymerase must rebind eventually if the DNA is to be elongated and to count as a final complete product. Thus, even in vivo, the value of θ must lie in the range of 0–1, thus establishing that the exonuclease contribution to fidelity of T7 DNA polymerase lies in the range of 190–210. For T7 polymerase at least, these correction terms are small and do not greatly affect the outcome of our analysis. The same cannot be said for Pol I (see below).

The cost of an error correction mechanism has been dicussed at length elsewhere (Fersht, 1985). For the 3′ → 5′ exonuclease of T7 DNA polymerase, we calculated the cost by using the kinetic parameters for a correctly base-paired DNA as follows:

$$\text{Cost} = 1 - \frac{\theta k_{off}^c + k_{p\rightarrow x}^c + k_p^c}{k_p^c} = 1 - \frac{0.05(0.2) + 0.2 + 250}{250} = 0.0008$$

This cost of less than 0.1% would be insignificant in vivo. Thus, the exonuclease provides a substantial increase in fidelity with a negligible cost in terms of wasted energy. In the absence of kinetic partitioning, where $k_{p\rightarrow x}$ and k_{off} would be the same for correct and incorrect DNA, the cost would be increased by an order of magnitude.

The repair rate by Klenow is limited by its exceptionally slow k_x of 0.003 s^{-1}. T7 DNA polymerase, on the other hand, has a 1000-fold faster repair rate of 2.3 s^{-1}, which is limited by its transfer rate, $k_{p\rightarrow x}$. In this context, the 0.1% cost calculated for T7 DNA polymerase must also be balanced against its intrinsic k_x of > 700 s^{-1}, whereas the 0.6% cost calculated for Klenow is balanced against an intrinsic k_x of 0.003 s^{-1}. It is not surprising that most of the available data on Klenow indicate the lack of any direct proofreading without dissociation of the DNA from the enzyme (Kuchta et al., 1988; Joyce, 1989). The dissociation rate constant is approximately 0.2 s^{-1} (as an average) while the rate of excision is 0.003 s^{-1}. Given the rate of polymerization on top of mismatch (0.01–0.000 02 s^{-1}), it is clear that the most probable event following misincorporation is the dissociation of the DNA from the enzyme (94–98%). Consequently, the exonuclease does not have time to act prior to dissociation of the DNA from the enzyme, and measurement of the excision rate must be governed by the kinetics of dissociation and reassociation of the enzyme–DNA complex. Rigorous calculations of selectivity for the exonuclease cannot be performed for Klenow on the basis of data published in Kuchta et al. (1988) because the fate of the DNA that dissociates from the enzyme without error correction has not been determined. In this case, the contribution from this pathway is substantial and must dominate the measurement of the excision rate.

Kuchta et al. (1988) have argued for an additional stage to the error correction mechanism that invokes the movement of the DNA from the polymerase site to the exonuclease site without going through a state capable of dissociating. This rather contrived mechanism was invented to explain the anomalous behavior of a few templates where the rates of excision were too slow to account for the observed turnover of dNTP to dNMP, and yet DNA molecules containing a mismatch failed to accumulate in solution in the presence of excess DNA. However, other explanations exist. For example, the more rapid binding of the DNA to the exonuclease site with selectivity toward the mismatched DNA (as observed here for T7 DNA polymerase), or the continued activity of the exonuclease toward mismatches while the polymerase site is occupied, could account for the data. In this respect, the Klenow fragment may exhibit properties not common to the Pol I holoenzyme and not shared by T7 DNA polymerase or other replicative enzymes. Alternatively, it is conceivable that the DNA visits the exonuclease site on the pathway toward dissociation from the enzyme. In any event, there is no compelling evidence to suggest an additional error correction mechanism, especially one that requires movement of the DNA without the potential for dissociation from the enzyme. We are left with the conclusion that the exonuclease site is only accessible to the DNA through the multiple equilibria involving the sliding of the DNA between the sites in the weak binding state [see Patel et al. (1991)], with opportunities for dissociation, movement to the polymerization site for the next incorporation, or movement to the exonuclease site according to the respective rate constants.

From experiments done with a synthetic cross-linked primer/template, Cowart et al. (1989) have suggested that a minimum of five bases must melt before excision can take place by Klenow. In the T4 and T7 DNA polymerases, they have shown that excision continues to within only one and two bases of the cross-link respectively, and they suggest that the two active sites in these polymerases might be much closer than in Klenow. However, the homology between T7 DNA polymerase and Pol I argues strongly against this assertion. The greater extent of excision by T4 and T7 DNA polymerases relative to Pol I can be directly correlated with the faster

exonuclease rates. Accordingly, the cross-linking experiments do not necessarily provide direct structural information about the active sites of the enzyme.

These major kinetic and mechanistic differences between the two polymerases can be attributed directly to their different functions in vivo. Because Klenow (as Pol I) functions primarily as a repair enzyme, it is not required to replicate long stretches of DNA within a short time constraint. This is evidenced by its slower polymerization rate as well as by its 30-fold lower processivity relative to T7 DNA polymerase. As a result, an efficient method of removing errors becomes less important.

A simple analysis based upon probabilities indicates that, given a polymer of length N and an error frequency e, the fraction of polymers containing an error can be calculated as $1 - (1 - e)^N$ (Galas et al., 1986). Since Pol I synthesizes polymers 50–100 base pairs in length, less than 1 out of 2000 polymers synthesized will contain an error if the error frequency is 10^{-6} (Kuchta et al., 1988). Since the contribution from the fidelity of the polymerase selectivity is sufficient to account for such an error frequency, a large contribution from the exonuclease would be unnecessary. In contrast, the highly processive T7 DNA polymerase must synthesize DNAs longer than 1500 bases per binding event. With an error frequency estimated $(0.5–6) \times 10^{-5}$ without the contribution of the 3′ → 5′ exonuclease (Wong et al., 1991), the corresponding fraction of products containing errors would be 1 per 10–150 polymers synthesized. The additional fidelity of 4×10^{-3}, contributed by its 3′ → 5′ exonuclease activity, significantly reduces this fraction to less than 1 per 40 000 polymers synthesized.

Acknowledgments

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Registry No. 25/36-mer, 130522-93-9; 26T/36-mer, 130522-96-2; $26T_2$/36-mer, 130522-95-1; $26T_3$/36-mer, 130522-94-0; exodeoxyribonuclease, 9068-32-0; DNA polymerase, 9012-90-2.

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