Threonine 79 Is a Hinge Residue That Governs the Fidelity of DNA Polymerase β by Helping to Position the DNA within the Active Site*

Mausumi Maitra‡, Andrew Gudzelak, Jr.§, Shu-Xia Li‡¶, Yoshihiro Matsumoto‡, Kristin A. Eckert‡, Joachim Jager‡‡, and Joann B. Sweasy¶ ¶‡‡

From the ‡Department of Therapeutic Radiology and Genetics, Yale University School of Medicine, New Haven, Connecticut 06520, the §Department of Biochemistry and Molecular Biology and The Jake Gittlen Cancer Research Institute, Pennsylvania State University College of Medicine, M. S. Hershey Medical Center, Hershey, Pennsylvania 17033, the ¶Department of Radiation Oncology and Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111, and the ¶‡‡School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

DNA polymerase β (pol β) is an ideal system for studying the role of its different amino acid residues in the fidelity of DNA synthesis. In this study, the T79S variant of pol β was identified using an in vivo genetic screen. T79S is located in the N-terminal 8-kDa domain of pol β and has no contact with either the DNA template or the incoming dNTP substrate. The T79S protein produced 8-fold more multiple mutations in the herpes simplex virus type 1-thymidine kinase assay than wild-type pol β. Surprisingly, T79S is a misincorporator mutant only when using a 3′-recessed primer-template. In the presence of a single nucleotide-gapped DNA substrate, T79S displays an antitrust phenotype when catalyzing DNA synthesis opposite template C and has similar fidelity as wild type opposite templates A, G, or T. Threonine 79 is located directly between two helix-hairpin-helix motifs located within the 8-kDa and thumb domains of pol β. As the pol β enzyme closes into its active form, the helix-hairpin-helix motifs appear to assist in the production and stabilization of a 90° bend of the DNA. The function of the bent DNA is to present the templating base to the incoming nucleotide substrate. We propose that Thr-79 is part of a hydrogen bonding network within the helix-hairpin-helix motifs that is important for positioning the DNA within the active site. We suggest that alteration of Thr-79 to Ser disrupts this hydrogen bonding network and results in an enzyme that is unable to bend the DNA into the proper geometry for accurate DNA synthesis.

Mammalian DNA polymerase β (pol β)† has quickly become one of the best studied polymerases because the gene for the enzyme was cloned (1, 2). The availability of multiple crystal structures of human and rat pol β, including those of the enzyme complexed with both of its substrates and the metal cofactor, has aided the investigation of the structure-function relationships of this enzyme (3–8).

pol β is a 39-kDa protein with both nucleotidyltransferase and 5′-deoxyribose phosphodiesterase activities (9, 10). Evidence has been provided for a role for pol β in both base excision repair and meiosis (11–13). There is no evidence that pol β functions in replication of the mammalian genome, but pol β has been shown to participate in DNA replication in *Escherichia coli* in the absence of DNA polymerase I (14). Mice that are completely deficient in pol β die at 18 days post-conception due to massive apoptosis of post-mitotic neurons, suggesting that pol β is essential for embryonic development (15, 16). The physiological DNA substrate for pol β is believed to be a small gap because it has been shown that pol β is processive on gaps of 6 bases or less and that the activity and fidelity of pol β are highest on a 1-bp gap with a 5′-phosphate (17, 18).

DNA polymerase β has a modular organization with an 8-kDa N-terminal domain connected to the 31-kDa C-terminal domain by a protease-hypersensitive hinge region. The N-terminal 8-kDa domain was originally characterized as a single-stranded DNA binding domain (19, 20). Subsequently, it was found to interact most efficiently with the 5′-phosphate of the downstream primer of the gapped DNA (21, 22). This interaction is mediated by a helix-hairpin-helix motif (HHH), which is found in several other DNA repair enzymes (4, 23, 24). Matsumoto and Kim (9) later demonstrated that pol β catalyzes removal of dRP from AP endonuclease-incised AP sites via β-elimination, as opposed to hydrolysis, and that this dRP lyase activity resides in the N-terminal 8-kDa domain of pol β. The DNA polymerase active site is found in the 31-kDa domain (25). pol β does not possess proofreading activity.

We have developed a genetic screen to identify the amino acid residues of pol β that are critical for fidelity (26, 27). Our screen is based upon the discovery that rat pol β substitutes for *E. coli* DNA polymerase I in DNA replication (14). We constructed a library of pol β mutants in which only the 8-kDa domain of the protein was mutated. By using our genetic screen, we isolated several mutator mutants of pol β, including one that is altered from Thr to Ser at position 79 (T79S). The T79S alteration is located in helix IV of the N-terminal 8-kDa domain of pol β and appears to be distant from the catalytic active site, having no contact with the substrate during catalysis, as shown in Fig. 1. Thr-79 is positioned directly between
two HhH motifs, HhH 1 and 2. HhH motif 1 interacts with the downstream oligonucleotide, and HhH 2 interacts with the primer strand in single nucleotide gapped DNA.

To elucidate the role of the Thr-79 residue in maintaining pol β fidelity, we have employed a transient state kinetic approach. The catalytic pathway of pol β is shown in Scheme 1. First, pol β binds to the DNA substrate, followed by binding to the dNTP substrate. After formation of the ternary complex of pol β, DNA, and dNTP, a conformational change occurs to produce an active complex (β*) that can catalyze DNA primer extension. After phosphodiester bond formation, pyrophosphate is released. Finally, pol β dissociates from the DNA substrate during the rate-limiting step. Mutator mutant proteins have been shown to alter kinetically the steps in the pathway of DNA polymerization (28, 29). This often leads to a decrease in fidelity. Our transient state kinetic results demonstrate that the interaction of the HhH domains of pol β with the downstream DNA and the primer of the DNA substrate is important for accurate DNA synthesis by pol β. Our findings suggest that movements of the HhH motifs are mediated by Thr-79. This movement results in presentation of the template within the active site of pol β and stabilization of the primer. We suggest that the placement of the template within the pol β active site and stabilization of the primer strand are critical determinants of DNA synthesis fidelity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media**—The strain BL21 DE3 was used for protein expression and has genotype F ompT hsdSB(rBmB) gal dcm (DE3). The strain FT334 is an HB101 derivative and was used to detect thymidine kinase mutations in the forward mutation assay. Its genotype is recA13 upp tdk (30), E. coli strains CSH5 F’(pro-lac), ara, thi, strA, FpyrAB, lacQZM15, trd36) (31) and MC1061 (lac37, lac127, araD139, araE139, lacIPOZY) were used in the M13 fidelity assays. Strain SC18-12 is derived from E. coli B/r and has the genotype recA178 polA12 wua155 trpE65 lon-11 suA1 (27). The SC18-12 strain was used in screening of a cDNA library of pol β mutants (27).

ET medium was E salts (32) supplemented with 0.4% glucose and 20 μg/ml tryptophan. Eglu medium is ET without tryptophan. Transformants were selected on Luria-Bertani agar (33) supplemented with 30 μg/ml chloramphenicol and 12 μg/ml tetracycline. Nutrient broth was used in the preparation of the DNA substrates were synthesized at the Keck Molecular Biology Center at Yale University.

**Template Presentation by DNA Polymerase β**

Expression and Purification of Mutant Enzymes—The cDNAs of pol β-wt and T79S were subcloned into the pET28a vector (Novagen), 3’ to a hexahistidine tag, resulting in fusion of the tag to the N termini of the proteins. These enzymes were expressed and purified as described previously (34), using a fast protein liquid chromatography-driven Ni²⁺ column (Hi-Trap chelating HR10/10, Amersham Biosciences). The protein was eluted with an imidazole gradient. This was followed by Hi-Trap SP column (Amersham Biosciences) where the protein was eluted with a NaCl salt gradient. Proteins were greater than 90% homogenous based on a Comassie Blue-stained SDS-PAGE. Concentrations of pol β proteins were based on an ε₂₆₀ = 21,200 M⁻¹ cm⁻¹ and a molecular mass of 40 kDa for His-tagged pol β.

**Identification of Mutator Mutants Using the Trp⁻ Reversion Assay**—The T79S mutant was identified in a genetic screen developed in our laboratory to isolate pol β mutator mutants (27). We used the Trp⁻ reversion assay to identify the mutator mutants from a library of random mutants constructed between nucleotides 1 and 300; this fragment encodes amino acids 1–100 of pol β. The library was constructed by mutagenic PCR and subcloning, as described (27). In our genetic screen, mutator mutants induce significantly more Trp⁻ revertants than cultures containing the pol β wild-type gene. The mutation of each pol β mutant was identified by the dyeoxy DNA sequencing method using Sequenase 2.0 (United States Biochemical Corp.) according to the manufacturer’s directions.

**Pre-steady-state Analysis**—Reactions were performed in which radiolabeled gapped DNA (300 ng 45X-22-22) was in 3-fold excess relative to pol β (100 nM). These reactions are referred to as burst experiments (29). The burst experiment was performed at saturating concentrations of dNTP while minimizing any enzyme inhibition, which only occurs with excess dNTP. Reactions were initiated by rapid mixing of the pol β/DNA and Mg-dNTP solutions (final concentration of MgCl₂ = 10 mM). At selected time intervals, the reactions were quenched with 0.5 M EDTA. The reactions products were separated as described below.

**Single Turnover Misincorporation Assays**—To elucidate the relative ability of the T79S enzyme compared with pol β-wt to incorporate correct and incorrect dNTPs into a primer-template, we determined the equilibrium dissociation constant for dNTP binding, Kᵣ, and the maximum rate of polymerization, kₚ,p, for correct and incorrect dNTPs for each enzyme. For both pre-steady-state and single turnover condition assays, reactions were conducted in buffer (50 mM Tris-Cl, pH 8.0) containing 2 mM dithiothreitol, 20 mM NaCl, and 10% glycerol. All concentrations given refer to the final concentrations after mixing. The kinetics of correct dNTP incorporation were determined under single turnover conditions using rapid chemical quench performed on a KinTek Instruments model RQF-3 rapid quench-flow apparatus (36) thermostatted at 37 °C. Single turnover kinetic experiments were performed under conditions where the enzyme concentration greatly exceeds the Kᵣ value for gapped DNA. Single turnover conditions were determined empirically to be a ratio of enzyme to DNA of 15:1 (data not shown) for T79S. These conditions allow binding of greater than 95% of the DNA substrate by pol β and thereby measure the rate of a single catalytic turnover of the enzyme. Reactions were conducted at 37 °C in 50 mM Tris, pH 8.0, 10 mM MgCl₂, 2 mM dithiothreitol, 10% glycerol, 50 mM 3²³P-dATP (5000 Ci/mmol, 10 mCi/ml) were purchased from New England Biolabs, Sigma, and Amersham Biosciences, respectively. 5-Bromo-4-chloro-3-indolyl-D-galactoside (X-gal) and 5-fluoro-2'-deoxyuridine were purchased from Sigma. The oligonucleotides used in the preparation of the DNA substrates were synthesized at the Keck Molecular Biology Center at Yale University.
The kinetics of misincorporation were determined manually under the above single turnover conditions. Reactions were performed by preincubating 750 mM enzyme with 50 mM primer-template at 37 °C for 1 min. Reactions were initiated by the addition of substrate, incubated for the indicated reaction times, and stopped by the addition of 0.5 mM EDTA. Substrate concentrations, substrate concentrations were typically 0–2 mM, and reaction times were 0–2700 s.

The reactions resulted in the addition of one dNTP onto the primer. The n (unextended) and n + 1 (extended by one nucleotide) DNA products were resolved on a 20% Sequal NE (American Bioanalytical) polyacrylamide gel. The bands were quantified by an Amersham Biosciences Storm 840 PhosphorImager to measure product formation as a function of time.

**Data Analysis**—The data were fit by nonlinear regression using the program Sigmaplot version 4.14 (Jandel Scientific). The data from burst experiments were fit to the equation \[ \text{rate} = A \times (1 - \exp(-k_{obs}t)) + \text{fidelity} \times k_{pol} \times dNTP \times K_d + \text{(dNTP)}, \] where A is the amplitude of the burst; \( k_{obs} \) is observed first-order rate constant for dNTP incorporation, and \( k_{pol} \) is the observed steady-state rate constant. Single turnover kinetic data were fit to the single exponential equation \[ \text{rate} = A \times (1 - \exp(-k_{obs}t)), \] where A is the amplitude, and \( k_{obs} \) is the observed first-order rate constant for dNTP incorporation. To obtain \( k_{pol} \), the equilibrium dissociation constant, and \( k_{pol} \), the maximum rate of polymerization, the data were fit to the hyperbolic equation \[ k_{obs} = k_{pol} \times dNTP \times K_d + \text{(dNTP)}, \] Fidelity values were calculated using the following equation, fidelity = \((\text{observed}(K_d)/k_{pol} - \text{correct}) + (k_{pol}/K_d)\text{incorrect} - k_{pol}/K_d\text{correct})\).

**Single Turnover Mispair Extension Assays**—These assays were performed as described above, in single turnover conditions, except the primer-template contained mispaired termini (35T-20G). The insertion of the next correct nucleotide, dGTP, was measured.

**M13mp2-based Reversion Assays**—The fidelity of pol β-wt and T79S was measured using two M13mp2-derived templates, each of which contain a 390-bp gap opposite the lacZ gene and were constructed as described (31). For gap-filling DNA synthesis, 0.1 pmol of gapped DNA was incubated with 20 pmol of rat pol β-wt or T79S in 30 µl of buffer containing 50 mM Tris-Cl, pH 8.0, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, 200 µg/ml bovine serum albumin, and 500 µM dNTPs at 37 °C for 1 h. The reactions were terminated by the addition of EDTA to a final concentration of 15 mM. An aliquot of each reaction was analyzed on a 0.8% agarose gel containing ethidium bromide to be certain that the gap was filled completely (31). In each case, the reaction products comigrated with a double-stranded nicked molecular size standard, indicating that the gap had been filled to completion within our limits of detection (90%). Aliquots of each reaction were then transcribed into the M13mp18 complementary strand, and polymerase fidelity was calculated from the ratios of mutant (blue) and nonmutant (colorless) plaques on CHS550 F indicator E. coli as described (31).

**HSV-th Forward Mutational Assay**—The T79S polymerase was used in the HSV-th forward mutational assay as reported previously (37, 38). The M13-th template contains a portion of the HSV1-thymidine kinase gene and is a substrate of DNA synthesis that was purified, and the polymerase-synthesized strand was rescued by hybridization to a gapped heteroduplex molecule. Mutation frequency was calculated as described by Eckert et al. (37) after transfection into the FT334 strain. To ensure independence of selected mutant colonies for the mutational spectra, FT334 cells were aliquoted into multiple tubes containing VBA broth and independently transfected into the M13C061 strain. In parallel, mutation frequency was calculated as described (31).

**DNA Binding Assay**—The dissociation constant \( K_d \) (DNA) was measured using a gel mobility shift assay (39). One nucleotide gapped (45X-22-22) and 3’-recessed (45X-22) oligonucleotides were prepared and annealed as described above. Fifteen protein concentrations ranging from 4 µM to 0.25 mM, expected to bracket the \( K_d \), were incubated with 0.1 mM DNA that had been radiolabeled in buffer containing 10 mM Tris, pH 7.5, 6 mM MgCl₂, 100 mM NaCl, 10% glycerol, and 0.1% Nonidet P-40. After a 10-min incubation at 20 °C, samples were loaded onto a 6% acrylamide nondenaturing gel with the current running at 300 V. After loading, voltage was reduced to 150 V, and the gel was run for 1 h. Fractions bound were determined by PhosphorImager quantitation of the gel using an Amersham Biosciences Storm 840 PhosphorImager. The apparent dissociation constant, \( K_d \), was derived from Sigmoid fitting of the fraction bound versus protein concentration with the equation, \[ Y = (m_1 \times x / (K_d + m_2)) + m_3, \] where m1 is a scaling factor, and m3 is the apparent minimum Y value (39).

**dRP Lyase Assay**—Three oligonucleotides Oligo 1, Oligo 2, and Oligo 3 (Fig. 2) were annealed and labeled with [γ-32P]dATP (3000 Ci/mmol) and reverse transcriptase. After precipitation in ethanol, the labeled oligonucleotides were dissolved in 10 mM HEPES, pH 7.5, and treated with uracil-DNA-glycosylase immediately before use. The uracil-DNA-glycosylase-treated substrate (10 pmol/assay) was incubated in 10 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl₂ with indicated amounts of polymerases at 25 °C for 15 min. The reaction was terminated by the addition of SDS (final 0.5%), and the unexcised dRP was stabilized by 100 mM NaBH₄. After addition of an equal volume of the formamide/dye solution, the samples were resolved by electrophoresis in a 8 x urea-containing 20% polyacrylamide gel. The gel was subjected to autoradiography with an x-ray film and also scanned with a Fuji BAS PhosphorImager for quantitation.

**RESULTS**

**T79S Confers a Mutator Phenotype to the SC18-12 E. coli Strain**—To confirm that the candidate T79S mutant (Fig. 1) we identified in our genetic screen possesses a true mutator phenotype, we compared the spontaneous mutation frequencies of this mutant of the 8-kb domain of pol β with that of the pol β-wt strain using the Trp⁺ reversion assay (27). We found that the T79S mutant has a spontaneous mutation frequency that is 8-fold higher than the pol β-wt strain.

**T79S Shows a Rapid Burst of Product Formation**—We purified the protein as described under “Experimental Procedures” (28). A pre-steady-state burst experiment to monitor dTTP incorporation was performed under conditions where 45X-22-22 DNA (Fig. 2) was in 3-fold excess of pol β. Fig. 3 demonstrates insertion of dTTP opposite A by T79S at 37 °C occurs via an initial fast phase \( (k_{obs} = 3.78 \text{ s}^{-1}) \) followed by a slower, linear phase with a rate constant of 0.2586 s⁻¹. The biphase nature of T79S is similar to WT pol β (29) and indicates that the rate-limiting step of the catalytic cycle occurs after phosphodiester bond formation.

**T79S Has An Intrinsic Antimutator Activity in Vivo**—The in vivo data suggested that T79S confers a mutator phenotype to the SC18-12 E. coli strain. One interpretation of these data is that the mutator phenotype is caused by T79S incorporating...
incorrect nucleotide substrates, as it substitutes for pol I in DNA replication. To test this hypothesis (28), we compared the fidelities of DNA synthesis of T79S and pol β-wt.

First, a single turnover kinetic assay (28, 29, 39) was used to quantify the relative rates of nucleotide misincorporation catalyzed by the T79S and pol β-wt proteins opposite templates C, G, A, and T using a synthetic 5'–32P-end-labeled single base gapped template (45X-22-22) as we have described previously (28, 29, 39). Single gapped substrate was chosen because it was shown to be the most likely physiological substrate for pol β (17, 18). In this assay the enzyme is in vast excess of DNA, thereby minimizing DNA binding effects. Fig. 4 shows a plot of the observed rate constants (k_{obs}) versus the concentration of dGTP, enabling us to obtain the maximum rate of polymerization, k_{pol}, and the equilibrium dissociation constant, K_d, for T79S. The k_{pol} and K_d rate constants were used to calculate the fidelities for pol β-wt and T79S. As shown in Table I, we observed that T79S possessed an increased fidelity compared with pol β-wt of 7-, 5-, and 6-fold when we tried incorporating the incorrect dCTP, dTTP, and dATP substrates, respectively, opposite template C. The increased discrimination of substrates exhibited by T79S mainly occurred at the level of ground state binding (K_d). For misincorporation of dTMP and dGMP opposite template T, misincorporations of dGMP, dAMP, and dAMP opposite template G and dAMP, dGMP, dCMP opposite template A, no significant differences in the fidelity of DNA synthesis were observed between T79S and pol β-wt (data not shown). These data suggested that T79S exhibited antimutator activity during DNA synthesis opposite template C.

**T79S Is Not a Frameshift Mutator at a Run of T Residues**—Although T79S was identified as a mutator in our in vivo screen, we found it to be an antimutator opposite template C, in the in vitro misincorporation assay. To determine whether T79S was a mutator for frameshifts, we employed a reversion assay that detects minus 1-base frameshifts at a TTTTT sequence or at 36 other non-repetitive sites within the lacZ gene (40). We obtained a spontaneous mutation frequency of 4.84 × 10^{-3} for the T79S protein in this frameshift reversion assay, which is 1.4-fold less than that of the pol β-wt enzyme which has a spontaneous mutation frequency of 6.7 × 10^{-3}. This demonstrates that the T79S protein is not a mutator for frameshift errors in a run of T residues.

**Forward Mutation Spectrum**—Next, we generated a forward mutation spectrum to assess all possible errors committed by the T79S protein. We employed the HSV-iden assay (37) that detects all types of errors including large addition and deletions and multiple mutations, in addition to frameshift and base substitution mutations. This assay employs a primed single-stranded DNA substrate for DNA synthesis. The mutation spectrum for T79S is shown in Fig. 5, and a summary of the data appears in Table II. The major difference between T79S and pol β-wt in this assay is that T79S produces 8-fold more multiple mutations than pol β-wt protein. This suggests that DNA synthesis by T79S results in multiple mutations, defined here as mutations within 15 bases of each other and assumed to be the result of one polβ binding event. The increased propensity of T79S to produce multiple mutations may account for...
its mutator phenotype in vivo.

The T79S mutation spectrum displays several unique characteristics relative to pol β-wt. The most frequent base substitution mutation observed for T79S is G to T, which is rarely observed for pol β-wt. The pol β-wt enzyme (37) produces mainly A to G mutations. A putative hot spot of T to C base substitution mutations occurs at position 203 of the HSV-tk target sequence. As shown in Fig. 5, two single T to C mutations were produced at position 203, and two other T to C substitutions also occurred at this site as part of two tandem multiple mutations (data not shown). T to C base substitutions have not been observed at position 203 for pol β-wt. A hot spot of 1-base deletion mutations occurs at position 147 after DNA synthesis by T79S; pol β-wt sometimes produces 1-base deletions at this site but not at the frequency of T79S.²

² K. A. Eckert, unpublished data.
The T79S Mutant Produces Mutations in the HSV-tk Forward Mutation Assay

| Error class | T79S | pol β- wt | T79S/pol β- wt |
|-------------|------|-----------|---------------|
| MF (obs)    | 27 (79) | 14 (86) | 2.0          |
| Single      | 19 (67) | 14 (83) | 1.4          |
| Multiple    | 4 (12) | 0.49 (3) | 8.2         |
| Base substitution | 7.4 | 3.2 | 2.3 |
| Frameshift  | 17.3 | 10.0 | 1.7 |

Error specificity of polymerases T79S and pol β- wt. Error frequency values are calculated using the estimated polymerase error frequency, EF. This analysis includes independent frameshifts for each polymerase and excludes frameshifts that occurred as a tandem error event. These data are taken from Opresko et al. (38) with permission. Includes TK mutants that have one or more errors that are greater than 15 bases apart.

A remarkable aspect of the T79S spectrum is the increase in 1-base insertion mutations, some of which occur at non-repetitive sequences. In analyzing the rates of 1-base frameshift mutations, we determined that in general the rate increases as the length of the homonucleotide run increases for both T79S and pol β- wt (37), as shown in Table III. However, the 1-base frameshift error rate for pol β- wt increased 4-fold as the repeat length increased from three to four nucleotides, although this was not the case for T79S; the highest rate of 1-base frameshift mutation was at nucleotide runs of three for this mutant. In fact, the T79S mutant commits 1-base frameshifts predominantly at runs of three Cs, having an 11.9-fold increased frequency over pol β- wt at these runs. The major hot spot of mutation at a run of three Cs is at position 147 of the HSV-tk target sequence. The slippage mechanism is usually responsible for 1-base frameshift mutations at homonucleotide runs, and this mechanism most likely operates in the case of T79S. However, because the majority of 1-base frameshifts at a run of three Cs occurs mainly at position 147, it is possible that the mechanism of mutagenesis at this site could be more complex than misalignment of the template. The fact that the run of C residues at position 147 is part of a pseudorepeat sequence lends support to this hypothesis. T79S produces a 3:1 ratio of 1-base deletions to insertions, whereas with pol β- wt the ratio of 1-base deletions to insertions is 10:1. A putative hot spot for insertion mutations is at position 199, where C is inserted within the run of Cs at this position. The most likely mechanism to account for this 1-base insertion is slippage. Other 1-base insertions were detected at other positions of the tk target. However, most of these insertion mutations were most likely not produced by the slippage mechanism, because these occur in non-runs and because the nucleotide that is inserted is not templated by the next base in the template. What is intriguing is that each of these insertions is preceded by the primer sequence 5’-CG.

The T79S Mutant Is a Misincorporation Mutator at Position 203 of the HSV-tk Sequence—We detected a putative hot spot of mutation of T to C at position 203 of the HSV-tk target, as described above. As shown in Fig. 5, two single T to C mutations were produced at position 203, and two other T to C substitutions also occurred at this site as part of a tandem multiple mutation (data not shown). The T to C mutation could result predominantly from direct misincorporation of dGTP opposite template T or from slippage because there is a C after template T at position 203. Therefore, we tested the hypothesis that the T79S protein misincorporates dGMP opposite template T at this site, using a single turnover kinetic assay with the 35T-19 (tk) primer-template, which corresponds to the exact DNA sequence at position 203. As shown in Table IV, T79S has a 5-fold decreased fidelity of misincorporation of dGMP opposite T at position 203, in the single turnover assay. The mechanistic basis for the decreased fidelity is mainly during the ground state binding, Kp, of the dGTP substrate. The T79S mutant has a low affinity for both correct (A) and incorrect (G) substrates at this site. On the other hand, T79S was found to have the same fidelity as the WT protein when misincorporating dGMP opposite T with the single base gapped substrate, 35T-19-15 (tk), having the same sequence as the gene at position 203, as shown in Table V. These data show that T79S has difficulty in discriminating dGTP versus dATP opposite template T when utilizing a 3’-recessed DNA substrate. The template utilized in by pol β in our E. coli genetic screen is most likely similar to a 3’-recessed template.

Next, we asked whether the misincorporation event of dGTP opposite template T at position 203 of HSV-tk sequence was due to a slippage-mediated mechanism in the 3’-recessed primer-template (35T-19) because there is a C after the template T. So we altered the C after template T at position 203 to A and performed the misincorporation experiment with dCTP opposite T using 35TGG-19 as primer-template. In this scenario, T, the templating base, would significantly “slip” out of the active site and allow G to become the templating base. dCTP would then be incorporated opposite G. As shown in Fig. 6, T79S produces significantly less n + 1 products compared with pol β- wt, which suggests that the misincorporation event at position 203 by T79S was not due to a slippage-mediated mechanism but was the result of a direct misincorporation event. T79S acts as a mutator using 3’-recessed primer-template as a substrate but behaves like an antimutator when the 3’ downstream sequence is present. To determine whether misincorporation by T79S is sequence context-specific, we repeated the experiment of misincorporation of dGMP opposite T with T79S in a totally different sequence context (45T-22). T79S showed a 38-fold decreased fidelity compared with pol β- wt, in misincorporating dGMP opposite template T, as shown in Table VI. Here also the mechanistic basis of decreased fidelity is mainly due to less discrimination than pol β- wt during ground state binding (Kp) of the dGTP substrate. However, in this new sequence context, T79S has a higher affinity for dGTP versus dATP when the templating base is T. These data suggest that T79S behaves as a low fidelity polymerase specifically when utilizing 3’-recessed DNA substrate. On the other hand, T79S was found to exhibit an antimutator phenotype with a 4-fold higher fidelity than pol β- wt using a single base gapped DNA substrate (45T-22-22) as shown in Table VII.

T79S Does Not Extend a Mispair—We also compared the ability of T79S to extend a dGMP:T mispair within the sequence context of position 203 of the HSV-tk target, using single turnover conditions with primer-templates 35T-20G and 35T-20A, respectively. As shown in Table VIII, the overall catalytic efficiency for the extension of the dGMP:T mispair is the same for the T79S and pol β- wt enzymes. However, the mechanism governing the extension of the mispair differs for T79S and pol β- wt. The T79S polymerase discriminates against mispair extension 10 times more than pol β- wt at the level of kpol and about 10 times less at the level of Kp. This indicates that the molecular basis for discrimination against the extension of mispairs is significantly altered for the T79S enzyme.

The Affinity of T79S for Gapped DNA Is Slightly Decreased Compared with pol β- wt—A gel mobility shift assay was conducted to estimate the affinity of pol β for single base gapped DNA and 3’-recessed DNA (data not shown). The results of 5 experiments were averaged and demonstrated that the T79S
The T79S mutant misincorporates dGTP opposite template T in a 3'-recessed DNA substrate

\[
\begin{array}{cccccc}
\text{k}_{\text{pol}} & K_a^a & \frac{k_{\text{pol}}}{K_{d}} & K_{d} & F(10^{3}f) & \\
\text{s}^{-1} & \mu M & & & \\
\beta\text{-WT} & & & & \\
\text{T:A} & 16.65 \pm 1.16 & 54.3 \pm 10.3 & 3.06 \times 10^5 & \\
\text{T:G} & 0.0115 \pm 0.0016 & 379.5 \pm 133.5 & 7.0 & 30.3 & 10 \\
\text{T79S} & & & & \\
\text{T:A} & 13.6 \pm 1.1 & 454.8 \pm 77.7 & 0.29 \times 10^5 & 15.15 & 2 \\
\text{T:G} & 0.0106 \pm 0.0012 & 699.7 \pm 147.6 & 1.5 & & \\
\end{array}
\]

\(^{a}\) Units are micromolar; 35T-19 (tk) primer-template was employed in this experiment.

\(^{b}\) The \(k_{\text{pol}}\) for correct (c) divided by incorrect (i).

\(^{c}\) The \(K_{a}\) for incorrect (i) dNTP divided by correct (c).

\(^{d}\) Fidelity \((F)\) was calculated as described under “Experimental Procedures.”

\(^{e}\) Kinetic constants for incorporation of dATP opposite T.

\(^{f}\) Kinetic parameters for misincorporation of dGTP opposite template T.

The T79S mutant misincorporates dGTP opposite template T with similar fidelity as WT in a single base-gapped substrate

\[
\begin{array}{cccccc}
\text{k}_{\text{pol}} & K_a^a & \frac{k_{\text{pol}}}{K_{d}} & K_{d} & F(10^{3}f) & \\
\text{s}^{-1} & \mu M & & & \\
\beta\text{-WT} & & & & \\
\text{T:A} & 7.384 \pm 0.93 & 24.6 \pm 6.25 & 3.0 \times 10^5 & 251.6 & 1.2 \\
\text{T:G} & 0.0282 \pm 0.001 & 112.1 \pm 16.08 & 4.6 & & \\
\text{T79S} & & & & \\
\text{T:A} & 4.349 \pm 0.363 & 40.6 \pm 7.15 & 1.07 \times 10^6 & 56.3 & 1.9 \\
\text{T:G} & 0.0073 \pm 0.0023 & 129.6 \pm 16.35 & 3.2 & & \\
\end{array}
\]

\(^{a}\) Units are micromolar; 35T-19–15 (tk) primer-template was employed in this experiment.

\(^{b}\) The \(k_{\text{pol}}\) for correct (c) divided by incorrect (i).

\(^{c}\) The \(K_{a}\) for incorrect (i) dNTP divided by correct (c).

\(^{d}\) Fidelity \((F)\) was calculated as described under “Experimental Procedures.”

\(^{e}\) Kinetic constants for incorporation of dATP opposite T.

\(^{f}\) Kinetic parameters for misincorporation of dGTP opposite template T.

**DISCUSSION**

The T79S mutant was identified by a genetic screen developed in our laboratory. T79S has an 8-fold increased Trp\(^+\) reversion frequency compared with pol \(\beta\)-wt. We purified the T79S protein and examined its ability to misincorporate nucleotides in vitro, using a 1-bp gapped DNA substrate. In these assays, T79S appeared to be an antimutator polymerase when incorporating nucleotides opposite templates C and T within putative sites with similar fidelity as WT in 3'-recessed DNA substrates. The T79S mutant had a 4-fold lower estimated affinity for 1-bp gapped DNA (45X-22-22) than pol \(\beta\)-wt. We also found that the affinity of T79S was 4-fold lower than that of pol \(\beta\)-wt for 3'-recessed primer-template (45X-22) in each of two experiments. This indicates that alteration of Thr to Ser at position 79 results in the protein having a slightly decreased affinity for DNA.

**dRP Lyase Assay**—Because T79S is located in the 8-kDa domain of pol \(\beta\) and this domain possesses dRP lyase activity (9, 41), we wanted to see whether the mutation of threonine to serine at position 79 affected the dRP lyase activity (9) of the polymerase. We found no change in dRP lyase activity as shown in Fig. 7, suggesting that Thr-79 is not essential for the dRP lyase activity of pol \(\beta\).

**FIG. 6.** T79S does not produce a slippage-mediated error at position 203 of HSV-\(tk\) sequence. 35TG-19 primer-template was employed in this experiment. Misincorporation of dCTP opposite template T at position 203 was performed under single turnover conditions as described earlier. Reactions were performed for 7 different reaction times (0, 2, 4, 8, 12, 20, and 30 min) with each of the 5 different concentrations of dCTP. The 1st lane is the 0-time point for each set of reactions. pol \(\beta\)-wt was found to produce significantly more \(n+1\) products than T79S, which are most likely due to slippage-mediated mechanism.
Template Presentation by DNA Polymerase β

**Table VI**

| h<sub>pol</sub> | K<sub>c</sub> | k<sub>pol(c)/k<sub>pol</sub>(i)</sub> | k<sub>pol(c)/K<sub>c</sub>(i)</sub> | k<sub>pol/K<sub>d</sub></sub> | F<sub>i</sub>×10<sup>3</sup><sub>f</sub> |
|---------------|-------------|---------------------------------|-------------------------------|--------------------------|------------------|
| s<sup>-1</sup> | μM         |                                 |                               |                          |                  |
| β-WT          |             |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 9.2985 ± 0.392 | 97.0 ± 11.26                   | 9.5 × 10<sup>4</sup>           | 61.7                     |
| T:G<sup>−</sup> | 0.0053 ± 0.00045 | 344.4 ± 69.17              | 15.4                          |
| T79S          |             |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 5.6304 ± 0.7443 | 326.7 ± 86.95                | 1.7 × 10<sup>4</sup>           | 1.6                      |
| T:G<sup>−</sup> | 0.0034 ± 0.00005 | 32.5 ± 0.22                    | 104.6                         |

<sup>a</sup> Units are micromolar; 45T-22 primer-template was employed in this experiment.

**Table VII**

| h<sub>pol</sub> | K<sub>c</sub> | k<sub>pol(c)/k<sub>pol</sub>(i)</sub> | k<sub>pol(c)/K<sub>c</sub>(i)</sub> | k<sub>pol/K<sub>d</sub></sub> | F<sub>i</sub>×10<sup>3</sup><sub>f</sub> |
|---------------|-------------|---------------------------------|-------------------------------|--------------------------|------------------|
| s<sup>-1</sup> | μM         |                                 |                               |                          |                  |
| β-WT          |             |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 12.2 ± 0.35 | 26.5 ± 2.12                     | 4.6 × 10<sup>5</sup>           | 7.0                      |
| T:G<sup>−</sup> | 0.0156 ± 0.003 | 238.5 ± 107.3                 | 65.4                          |
| T79S          |             |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 13.8 ± 1.93 | 25.6 ± 11.6                    | 5.4 × 10<sup>5</sup>           | 31.2                     |
| T:G<sup>−</sup> | 0.004 ± 0.0005 | 230.8 ± 79.7                  | 17.3                          |

<sup>a</sup> Units are micromolar; 45T-22–22 primer-template was employed in this experiment.

**Table VIII**

| Enzyme | Terminal base pairs | h<sub>pol</sub> | K<sub>c</sub> | k<sub>pol(c)/k<sub>pol</sub>(i)</sub> | k<sub>pol(c)/K<sub>c</sub>(i)</sub> | k<sub>pol/K<sub>d</sub></sub> | F<sub>i</sub>×10<sup>3</sup><sub>f</sub> |
|--------|---------------------|---------------|-------------|---------------------------------|-------------------------------|--------------------------|------------------|
| β-WT   |                     | s<sup>-1</sup> | μM         |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 2.4 ± 0.3 | 320.5 ± 68.7                | 7332            |
| T:G<sup>−</sup> | 0.05 ± 0.005 | 341.4 ± 92.0              | 146             |
| T79S   |                     |               |             |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 6.3 ± 0.13 | 967.9 ± 33.4               | 6457            |
| T:G<sup>−</sup> | 0.01 ± 0.0007 | 88.4 ± 15.6              | 147             |

<sup>a</sup> Units are micromolar; 35T-20A (tk) and 35T-20G (tk) primer-templates were employed in this experiment.

**Table VII**

| Enzyme | Primer-template used | h<sub>pol</sub> | K<sub>c</sub> | k<sub>pol(c)/k<sub>pol</sub>(i)</sub> | k<sub>pol(c)/K<sub>c</sub>(i)</sub> | k<sub>pol/K<sub>d</sub></sub> | F<sub>i</sub>×10<sup>3</sup><sub>f</sub> |
|--------|----------------------|---------------|-------------|---------------------------------|-------------------------------|--------------------------|------------------|
| β-WT   | 35T-20A (tk)         | s<sup>-1</sup> | μM         |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 2.4 ± 0.3 | 320.5 ± 68.7                | 7332            |
| T:G<sup>−</sup> | 0.05 ± 0.005 | 341.4 ± 92.0              | 146             |
| T79S   | 35T-20G (tk)         |               |             |                                 |                               |                          |                  |
| T:A<sup>+</sup> | 6.3 ± 0.13 | 967.9 ± 33.4               | 6457            |
| T:G<sup>−</sup> | 0.01 ± 0.0007 | 88.4 ± 15.6              | 147             |

<sup>a</sup> Units are micromolar; 45T-22–22 primer-template was employed in this experiment.

<sup>b</sup> The k<sub>pol</sub> for correct (c) divided by incorrect (i).

<sup>c</sup> The K<sub>c</sub> for incorrect (i) dNTP divided by correct (c).

<sup>d</sup> Fidelity (F) was calculated as described under “Experimental Procedures.”

<sup>e</sup> Kinetic constants for incorporation of dATP opposite T.

<sup>f</sup> Kinetic parameters for misincorporation of dGTP opposite template T.

different sequence contexts, and was as accurate as pol β-<wbr/>wt opposite templates A and G, respectively. These data indicate that the mutator phenotype of T79S we detected in our E. coli screen was not a result of direct incorporation by T79S in a single nucleotide gap. To clarify whether T79S possessed inherent mutator activity, we employed the HSV-<wbr/>tk forward mutation assay. T79S was found to induce an 8-fold higher frequency of multiple mutations than pol β-<wbr/>wt in the forward assay, which was consistent with the mutator phenotype we detected originally using our genetic screen. Upon close inspection of the mutation spectrum obtained from copying the HSV-<wbr/>tk target with T79S, we found that T to C transitions were frequent at position 203 of this target, suggesting that T79S frequently misincorporated dGMP opposite template T at position 203. To determine whether this was the case, we prepared a 3<sup>′</sup>-recessed primer-template with the identical sequence context present at position 203 of the tk target, and we performed a misincorporation assay. We used a 3<sup>′</sup>-recessed primer-template because this type of DNA substrate best mimics the single-stranded DNA template used in the HSV-tk assay. In accordance with the mutation spectrum of T79S, we found that this enzyme has a 5-fold lower fidelity of DNA synthesis for insertion of dGMP opposite T using a DNA substrate with the sequence context of position 203 of the tk gene (35T-19 tk)). Next, we asked if T79S was able to misinsert dGMP opposite T using a 3<sup>′</sup>-recessed DNA substrate and that perhaps sequence context was important for the misincorporation to occur. To test this hypothesis, we examined misincorporation of dGMP opposite T using a 3<sup>′</sup>-recessed DNA primer-template with a sequence context that was unrelated to position 203 of the tk target sequence, and we found that T79S had a 38-fold lower fidelity than pol β-<wbr/>wt. However, when utilizing a single nucleotide gapped DNA substrate, T79S had a higher fidelity than pol β-<wbr/>wt. Therefore, we conclude that the
T79S polymerase variant is a misincorporation mutator when catalyzing DNA synthesis in the presence of a 3' recessed DNA substrate. The tk mutation spectrum also revealed that T79S produces a much higher amount of 1-base insertion mutations than pol -wt. The majority of these mutations occur at a run of C residues, suggesting that the slippage mechanism can account for these mutations. These data show that when Thr-79 is altered to Ser, the primer DNA has acquired a propensity to slip within the active site. Taken together, our results show that the nature of the interaction of pol -wt with the DNA is important for accurate DNA synthesis. Our results also demonstrate that the HhH domains of pol -wt are critical for accurate DNA synthesis.

The Rate-limiting Step of T79S Is after Phosphodiester Bond Formation— Pre-steady-state burst analysis for T79S protein shows a biphasic kinetic profile (Fig. 3) just like pol -wt (29) indicating that the rate-limiting step occurs after phosphodiester bond formation and is not changed due to its mutation to Ser.

T79S Has an Intrinsic Antimutator Activity When Using Single Nucleotide Gapped DNA—Single base gapped substrate is a preferred physiological substrate for the pol enzyme. We used single turnover kinetics to characterize all possible misincorporation events opposite A, T, C, and G in single nucleotide gapped DNA. The T79S mutant exhibited fidelity similar to pol -wt protein opposite A and G, respectively, but showed a surprisingly higher fidelity than pol -wt when incorporating nucleotides opposite templates C and T (Tables I and VII). For incorporation opposite template C in single nucleotide gapped DNA, the molecular basis of discrimination appears to be more dependent on ground state binding (\(K_d\)) than on the pre-catalytic conformation change or chemical steps of the reaction (\(k_{pol}\)). The pattern of higher \(K_d\) values for the incorrect incorporations opposite template C tested is striking (Table I). This suggests that the mutant enzyme is capable of discriminating correct versus incorrect nucleotides at the level of ground state binding. Because Thr-79 is far away from the active site and has no direct contact with the substrate or the DNA, this finding raises the possibility that remote residues, like Thr-79, may be involved directly or indirectly in the recognition and binding of nucleotide substrates.

T79S Is Altered Mainly in Its Discrimination Ability during Ground State Binding on 3'-Recessed DNA— T79S appeared to induce a higher frequency of T to C transitions at position 203 of the HSV-tk gene, suggesting that it frequently misinserted dGMP opposite T at this position. Using a 3'-recessed template that was identical to position 203, we showed that T79S misincorporated dGMP opposite T at this site more often than pol -wt. It appeared that this was a direct misincorporation event and was not due to a slippage-mediated mechanism (Fig. 6). We also demonstrated that T79S misincorporated dGMP opposite T using a 3'-recessed DNA sequence unrelated to the tk sequence at position 203. In both cases, T79S was less able than pol -wt to discriminate between the correct and incorrect dNTP substrates during ground state binding. In fact, when catalyzing DNA synthesis in the presence of the 3'-recessed DNA substrate unrelated to position 203, T79S preferred to insert dGMP versus dAMP opposite template T. We conclude that when Thr-79 is altered to Ser, the fidelity of DNA synthesis is dependent upon the structure of the DNA. This suggests that the geometry of the nucleotide binding pocket and/or the active site of the T79S enzyme in the presence of 3'-recessed DNA is different from that of pol -wt.

T79S Commits 1-Base Insertion Mutations— The ratio of single base deletions to insertions is much lower for T79S than the WT enzyme. Most of the insertions produced by T79S are at a run of three C residues in the tk target, suggesting that they occur by slippage of the primer. This suggests that alteration of
Thr-79 to Ser results in an inability of the enzyme to stabilize the primer within the active site. The other insertions cannot be accounted for by the slippage mechanism, because they occur in non-repetitive sequences and are not templated. This indicates that the non-run insertions may be mediated by some type of non-templated addition. What is curious is that the primer sequence preceding the insertion is in every case 5′-CG. We suggest that the 5′-CG might form a structure that is conducive to a non-templated addition of a nucleotide, especially in the absence of stabilization of the primer strand within the T79S active site.

Position of T79S in the 8-kDa Domain May Affect Catalysis—The HhH is a widespread motif involved in non-sequence-specific DNA binding. Most of the HhH motifs function as DNA-binding modules. However, some of them mediate protein-protein interactions or have acquired enzymatic activity by incorporating catalytic residues. For example, this motif in pol β has dRP lyase activity. The sequence and structural studies of different HhH-containing proteins show that most of the HhH motifs are integrated as a part of a five-helical domain. It typically consists of two consecutive HhH motifs that are linked by a connector helix and that display pseudo-2-fold symmetry. Most HhH domains are found to possess a conserved hydrophobic core and show clear structural integrity to be recognized as a distinct protein fold.

DNA polymerase β has two HhH motifs. One HhH motif is present within the 8-kDa domain of pol β, is composed of residues 1–81, and interacts with the 5′-phosphate of the downstream primer of a single nucleotide gapped DNA substrate. This interaction is important for the catalytic efficiency and fidelity of pol β (18, 42). The second HhH motif interacts with the primer. T79S is located within the hinge region of helix 4 of the N-terminal 8-kDa domain of pol β, and in this location it is directly between both of the HhH motifs of pol β. The hinge region in which Thr-79 is located appears to participate in the movement of the two HhH motifs.

pol β undergoes a conformational change from an open to a closed form after the binding of the dNTP substrate and before phosphodiester bond formation. This change results in the alignment of the primer, template, dNTP substrate, and catalytic residues of the protein in an optimum geometric configuration for phosphodiester bond formation. Amino acid residues important for catalysis and fidelity, including Arg-283, Met-282, and Phe-272, are not poised for catalysis in the open conformation of the enzyme. However, once the enzyme assumes the closed conformation, these residues are brought into proper geometry for catalysis and for monitoring the fidelity of the chemical step (29, 43). Residues Arg-283 and Phe-272 form hydrogen bonds and van der Waals contacts with the templating nucleotide in the closed conformation. For T79S, our data indicate that, like WT, once the closed conformation is assumed in the presence of incorrect substrate, the rate of catalysis of nucleotidyl transfer is quite slow. Thus, T79S is a mutator when catalyzing DNA synthesis from a 3′-recessed primer-template mainly because it cannot discriminate correct from incorrect substrates during ground state binding.

Examination of the crystal structure of pol β complexed with DNA and deoxy-CTP (Protein Data Bank Code 1BPY) shows that in the closed conformation the DNA is bent nearly 90°. In interacting with DNA, the HhH motifs of pol β in the 8-kDa and thumb domains appear to push the templating base in the direction of the nucleotide binding pocket and active site, as shown in Fig. 1. This bent conformation of the DNA most likely results in template presentation and primer stabilization that is required for accurate discrimination of dNTP substrates during their ground state binding. The HhH motifs also stabilize the sharp bend formed in the pol β-gapped DNA structure.

We suggest that Thr-79 is a residue that participates in the movement of both HhH motifs and/or functions in the stabilization of these motifs in the closed conformation. Our data show that when Thr-79 is altered to Ser, the enzyme is much less accurate than pol β-wt in the absence of the downstream primer and produces 1-base insertion mutations much more frequently than pol β-wt. The mechanistic basis for the decreased fidelity during misincorporation is loss of the ability to discriminate correct from incorrect dNTP substrates during the initial binding of these substrates to the polymerase. These data are consistent with the interpretation that in the absence of the downstream primer, the HhH motifs are unable to function to position properly the template and primer within the active site. Thr-79 Oγ-1 forms two hydrogen bonds with the main chain oxygens of Glu-75 and Lys-81, which belong to helices D and E of pol β as shown in Fig. 8. These interactions may help to stabilize the orientation or relative angle between the two α-helices and, in turn, the width of the active site cleft. The N-terminal end of the helix D carries residues (e.g. Lys-68) that form important interactions with the upstream DNA primer. A serine in position 79 may have slightly different rotamer preferences than a Thr, and the position for Oγ-1 seen in the wild-type enzyme may not be equally populated in the mutant. We suggest that improper template presentation results in active site geometry that can more easily accommodate the incorrect dNTP substrate.

Conclusions—In summary, we have identified a mutator mutant of pol β that is altered at residue 79, from Thr to Ser and has no direct contact with either the DNA or dNTPs. This suggests a view of enzyme function in which the residues outside the immediate areas of substrate binding and catalytic activity are responsible for the fine-tuning of polymerase function, including substrate specificity. Our data suggest that the precise positioning of the DNA template and primer into the active site is critical for maintaining the fidelity of DNA synthesis. Our data also suggest that Thr-79 participates in a hydrogen bonding network that acts to position the DNA within the active site and that this positioning is critical for the fidelity of pol β.

Acknowledgments—We thank Thomas Kunkel (National Institute of Environmental Health and Safety, Research Triangle Park, NC) for all bacteriophages and their corresponding strains for M13mp2-based reverversion assays. We also thank Amit M. Shah for frequent problem solving and Karen Anderson for helpful discussions of our kinetic studies.

REFERENCES
1. Abbotts, J., Sengupta, D. N., Zou, G., and Wilson, S. H. (1988) J. Biol. Chem. 263, 15094–15103
2. Price, A., and Lindahl, T. (1991) Biochemistry 30, 8631–8637
3. Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Science 264, 1891–1903
4. Pelletier, H., and Sawaya, M. R. (1996) Biochemistry 35, 12778–12787
5. Pelletier, H., Sawaya, M. R., Waffle, W., Wilson, S. H., and Kraut, J. (1996) Biochemistry 35, 12742–12761
6. Pelletier, H., Sawaya, M. R., Waffle, W., Wilson, S. H., and Kraut, J. (1996) Biochemistry 35, 12762–12777
7. Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Science 264, 1930–1935
8. Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) Biochemistry 36, 11205–11215
9. Matsumoto, Y., and Kim, K. (1995) Science 269, 699–702
10. Rein, D. C., Recuero, A. J., Reed, M. P., and Meyer, R. (1990) in The Eukaryotic Nucleus: Molecular Biochemistry and Macromolecular Assembly (Strauss, P. R., and Wilson, S. H., eds), pp. 95–123, Telford Press, Caldwell, NJ
11. Soehi, R. W., Horton, J. K., Kuhn, R., Gu, H., Singhal, R. K., Prasad, R., Rajevsky, K., and Wilson, S. H. (1996) Nature 379, 183–186
12. Plug, A. W., Clairmont, C. A., Sapi, E., Ashley, T., and Sweasy, J. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1327–1331
13. Clairmont, C. A., Narayanan, L., Sun, K.-W., Glazer, P. M., and Sweasy, J. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9580–9585
14. Sweasy, J. B., and Loeb, L. A. (1992) J. Biol. Chem. 267, 1407–1410
15. Gu, H., Marth, J., Orban, P., Mosmann, H., and Rajevsky, K. (1994) Science
Template Presentation by DNA Polymerase β

16. Sugo, N., Aratani, Y., Nagashima, Y., Kubota, Y., and Koyama, H. (2000) EMBO J. 19, 1397–1404
17. Singhal, R. K., and Wilson, S. H. (1993) J. Biol. Chem. 268, 15906–15911
18. Chagovetz, A. M., Sweasy, J. B., and Preston, B. D. (1997) J. Biol. Chem. 272, 27561–27564
19. Prasad, R., Kumar, A., Widen, S. G., Casas-Finet, J. R., and Wilson, S. H. (1993) J. Biol. Chem. 268, 22746–22755
20. Prasad, R., Beard, W. A., and Wilson, S. H. (1994) J. Biol. Chem. 269, 18096–18101
21. Casas-Finet, J. R., Kumar, A., Morris, G., Wilson, S. H., and Karpel, R. L. (1991) J. Biol. Chem. 266, 19618–19625
22. Prasad, R., Beard, W. A., Strauss, R. P., and Wilson, S. H. (1996) J. Biol. Chem. 271, 15263–15270
23. Doherty, A. J., Serpel, L. C., and Ponting, C. P. (1996) Nucleic Acids Res. 24, 2488–2497
24. Mullen, G. P., and Wilson, S. H. (1997) Biochemistry 36, 4713–4717
25. Beard, W. A., and Wilson, S. H. (2000) Mutat. Res. 460, 231–244
26. Sweasy, J. B., and Loeb, L. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4626–4630
27. Washington, S. L., Yoon, M. S., Chagovetz, A. M., Li, S., Clairmont, C. A., Preston, B. D., Eckert, K. A., and Sweasy, J. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1321–1326
28. Li, S., Vaccaro, J., and Sweasy, J. B. (1999) Biochemistry 38, 4800–4808
29. Shah, A. M., Li, S.-X., Anderson, K. S., and Sweasy, J. B. (2001) J. Biol. Chem. 276, 10824–10831
30. Eckert, K. A., and Drinkwater, N. R. (1987) Mutat. Res. 178, 1–10
31. Bebenek, K., and Kunkel, T. A. (1995) Methods Enzymol. 262, 217–232
32. Vogel, H. L., and Bonner, D. M. (1956) J. Biol. Chem. 218, 97–106
33. Miller, J. H. (1952) A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
34. Kosa, J. L., and Sweasy, J. B. (1999) J. Biol. Chem. 274, 3853–3858
35. Sweasy, J. B., and Yoon, M. S. (1995) Mol. Gen. Genet. 245, 217–224
36. Johnson, K. A. (1995) Methods Enzymol. 249, 38–61
37. Eckert, K. A., Hile, S. E., and Vargo, P. L. (1997) Nucleic Acids Res. 25, 1450–1457
38. Opresko, P. L., Sweasy, J. B., and Eckert, K. A. (1998) Biochemistry 37, 2111–2119
39. Kosa, J. L., and Sweasy, J. B. (1999) J. Biol. Chem. 274, 35866–35872
40. Bebenek, K., Joyce, C. M., Fitzgerald, M. P., and Kunkel, T. A. (1990) J. Biol. Chem. 265, 13878–13887
41. Beard, W. H., and Wilson, S. H. (1995) Methods Enzymol. 262, 98–107
42. Wilson, S. H. (1998) Mutat. Res. 407, 203–215
43. Werneburg, B. G., Ahn, J., Zhong, X., Hondal, R. J., Kraynov, V. S., and Tsai, M. D. (1996) Biochemistry 35, 7041–7050