Hinge Residue Ile260 of DNA Polymerase β Is Important for Enzyme Activity and Fidelity†

Daniela Starcevic,‡ Shibani Dalal,§ and Joann Sweasy*,§

Departments of Genetics and Therapeutic Radiology and Program in Microbiology, Yale University School of Medicine, New Haven, Connecticut 06520

Received September 22, 2004; Revised Manuscript Received December 15, 2004

ABSTRACT: DNA polymerases ensure efficient insertion of the correct dNTP into the DNA substrate. They have evolved mechanisms for discriminating among very similar dNTP substrates. DNA polymerase β is a repair polymerase that provides a model system for a direct study of insertion fidelity. In this study, we examined the role of hinge residue Ile260 of the rat Polβ on enzyme activity and accuracy. We changed residue I260 to every other amino acid residue and used genetic screens to assess the activity and fidelity of the resulting mutants. The I260D, -E, -K, -N, and -R mutants are significantly less active than wild-type Polβ. Interestingly, I260H and I260Q are active but exhibit mutator activity. This suggests that the nonpolar nature of residue 260 is important for maintaining the activity and fidelity of Polβ. We employ molecular modeling as an aid in explaining the observed phenotypes and propose a mechanism whereby the positioning of the DNA substrate in the enzyme and within the surface of the hinge may be a key player in forming an optimal active site for phosphodiester bond formation between Watson−Crick base pairs.

The survival of organisms largely depends on the accurate transmission of genetic information. This faithful transmission requires not only accuracy in DNA replication and precision in chromosome distribution but also the ability to survive spontaneous and induced DNA damage. Eukaryotic cells have established and maintained a variety of DNA repair pathways. Base excision repair (BER)[1] is one such pathway geared at repairing single-base damage. This process is responsible for repairing about 10 000 lesions per cell per day which are mostly generated by reactive oxygen species and alkylating agents (*1*−*3*). Repair is initiated with the event of damage recognition by a specific DNA glycosylase which excises the damaged base (*4*). The phospate backbone is then cleaved by an apurinic/apyrimidinic endonuclease (*5, 6*), leaving a 3′-OH moiety ready for nucleophilic attack. In most BER events, Polβ fills in the missing nucleotide. In short patch BER, where a single nucleotide is added, Polβ provides an additional function: it removes the 5′-deoxyribose phosphate using its dRP lysase activity (*7*). This sequence of events provides a substrate for a ligase which can now seal the nick, thus completing repair (*8*). Polβ does not have intrinsic nuclease or proofreading activities, and it is therefore thought that in an in vitro system, where no extrinsic proofreading nucleases are available, fidelity depends primarily upon the proper alignment among the enzyme, the DNA substrate, and the incoming dNTP (*9*−*11*). This lack of proofreading activity makes Polβ an ideal system for studying fidelity directly. Fidelity studies using Polβ as a model system are further facilitated by the fact that several structures of Polβ in complex with its substrate(s) are available (*12*), and the fact that Polβ is a small enzyme of 39 kDa, making it easy to purify and study.

Template-directed synthesis requires that the polymerase choose the correct nucleotide within a pool of very similar substrates. Many studies have focused on the active site of Polβ. However, it is becoming increasingly obvious that sites other than the active site and which have no apparent direct contact with either the DNA substrate or the incorporating dNTP are essential for fidelity (*9*−*11, 13*). These active site distant regions have been proposed to be implicated in changing the conformation of the enzyme to optimally position the 3′-OH of the primer, the template, and the dNTP for efficient phospodiester bond formation. This alignment appears to be essential for "error-free" synthesis by Polβ.

The hinge is a hydrophobic region in the carboxy-terminal domain of Polβ. Isoleucine 174, threonine 196, and tyrosine 265 form the outside lining of the hinge, whereas leucine 194, isoleucine 260, and phenylalanine 272 line the inside of this region (*12*). Structural information on Polβ (*12*) has suggested that this enzyme can exist in either an open or a closed conformation. The open conformation is observed in the absence of the dNTP, whereas the closed conformation is the result of dNTP binding, positioning the enzyme for phosphodiester bond formation. Figure 1 shows the position of the hinge residues in the open (panel A) and closed (panel B) conformations of the enzyme resulting from a movement

† D.S. was partially supported by The Yale and Bristol-Myers Squibb Research Educational Alliance grant. This research was supported by Grant CA80830 (to J.S.) from the National Cancer Institute. J.S. is a Donaghue Investigator.

* To whom correspondence should be addressed: Department of Therapeutic Radiology, Yale University School of Medicine, 333 Cedar St., P.O. Box 208040, New Haven, CT 06520. Phone: (203) 737-2626. Fax: (203) 785-6309. E-mail: joann.sweasy@yale.edu.

‡ Program in Microbiology.

§ Departments of Genetics and Therapeutic Radiology.

[1] Abbreviations: BER, base excision repair; Polβ, DNA polymerase β.

10.1021/bi047956x CCC: $30.25
Published on Web 02/12/2005

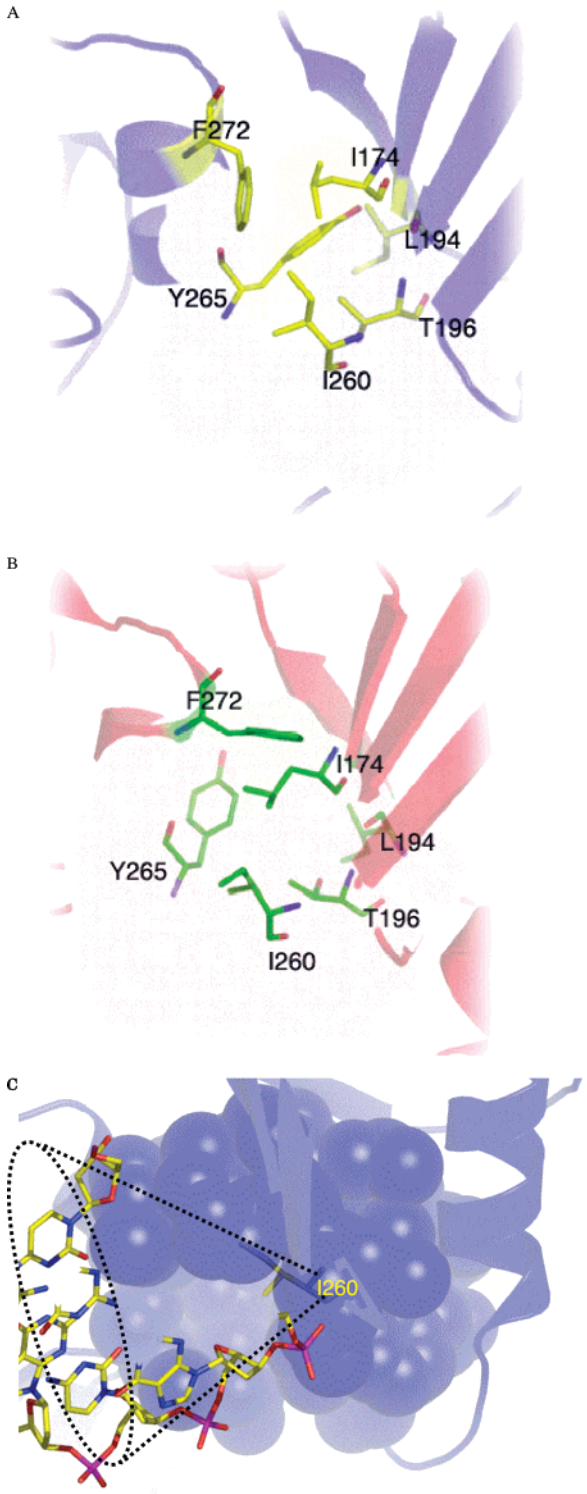

FIGURE 1: Hinge residues move upon closing of DNA polymerase β. Side chains of Ile174, Leu194, Thr196, Ile260, Tyr265, and Phe272 are shown to emphasize the difference in their position in the open and closed conformations of the enzyme. (A) Amino acid residues of the hinge in the open (binary) Polβ complex of the enzyme and DNA substrate. (B) Position of the amino acid side chains in the closed (ternary) complex of Polβ consisting of the enzyme, DNA substrate, and incoming dNTP. Panels A and B were generated using PyMol to visualize Swiss-Model energy-minimized structures of Polβ from Protein Data Bank entries 1bpx (binary) and 1bpy. (C) Illustration of the DNA substrate position relative to I260 in the open conformation of Polβ. Spheres denote an area of 8 Å around Ile260 in the open conformation of Polβ (generated from PDB entry 1bpx). The DNA substrate is shown as sticks. The dashed lines outline the cone that the hinge comprises.

of the carboxy-terminal region of the enzyme by 12 Å relative to the palm domain. The differences in individual hinge residue positioning are quite obvious, depicting the dramatic changes in the enzyme as it accommodates its substrates in the active site and moves from an open to a closed conformation.

Among the variants of Polβ with compromised ability to discriminate between the correct and incorrect nucleotide during incorporation, hinge mutants have been identified. Y265H is one such mutator mutant which not only induces an increased mutation frequency when compared to the wild-type enzyme but also has a kinetic pathway of polymerization different from that of the wild type. The rate-limiting step of Y265H is during the process of phosphodiester bond formation or earlier, whereas this step for the wild-type enzyme is at the point of dissociation from the DNA (*13*, *14*). Y265C also has also been classified as a mutator mutant (*15*, *16*). Two other interesting variants of this residue have been identified. Y265W and Y265F are both impared in their ability to choose the correct substrate when filling a single-nucleotide gap. Interestingly, these mutants are also able to extend mispaired termini (*17*). F272 is another hinge residue that is also responsible for an aspect of enzyme activation where the closing of the enzyme moves it to disrupt the interaction between Asp192 and Arg258. This allows Asp192 to coordinate the magnesium ions and promote activation. The F272L mutant has been studied and, not surprisingly, is impaired in discriminating between the correct and incorrect nucleotide (*18*).

The data pointing to the pivotal role that hinge residues play in the overall fidelity of polymerization by Polβ have prompted us to investigate the role of another hinge residue, namely, I260, in the activity and fidelity of Polβ. This residue is located in the pit of the hinge. We reasoned that any changes in the local environment of this residue could domino out toward the surface of the hinge in the plane where the DNA substrate is positioned. In fact, the DNA substrate sits several planes directly above the hinge with residue 260 in the center. This is shown in Figure 1C where the spheres represent residues 8 Å around I260. Residue I260 itself is barely visible because it is buried in the bottom of the hinge region. For purposes of visualization, we liken the hinge to a cone where residue 260 is at the tip of the cone while the flat surface of the cone represents the area where the DNA substrate is positioned within the enzyme (as outlined by the dashed lines in Figure 1C). It is also of interest to note that the low-fidelity I260M variant of Polβ has been found to be associated with prostate cancer, further pointing to the pivotal role that the hinge and this residue might be playing in the activity and fidelity of Polβ (S. Dalal, manuscript in preparation).

Here we present data showing that when position 260 is altered to Lys, Arg, Asn, Asp, or Gln instead of Ile, the resulting enzymes are severely impaired in their activity. Interestingly, all of these substitutions change the chemical nature of this residue. Furthermore, the I260H and I260Q variants maintain wild-type levels of activity but are mutator mutants. These data suggest that changing the chemical nature of this residue impairs the enzyme's activity or accuracy. We propose that subtle chemical differences in this active site distant local environment strongly affect the enzyme's ability to accurately perform its function.

MATERIALS AND METHODS

Bacterial Strains and Media. Strain SC18-12 was used in the in vivo complementation assays and Trp^+ reversion assays, and it has the *recA718 polA12 uvrA155 trpE56 lon11 sulA1* genotype. The pBADHis (Invitrogen) and pET28a-(+) (Novagen) vectors containing wild-type or mutant rat Polβ were propagated in DH5αMCR which has the *mcrA d(mrr-hsdRMS-mcrBC) f80dlacZdM15 (lacZYA-argF)U169 deoR recA1 endA1 phoA supE44 thi-1 gyrA96 relA1* genotype. Strain BL21DE3 was used for protein expression; it has the *F-ompT hsdSB(rB-mB-) gal dcm* (DE3) genotype.

Nutrient agar (NA) was Difco nutrient agar with 4 g/L NaCl. Nutrient broth (NB) and Luria broth (LB) were prepared according to the manufacturer's instructions (Difco). ET medium was E salts (Vogel and Bonner) supplemented with 0.4% glucose and 20 μg/mL Trp. Eglu medium is ET without Trp. Antibiotics were used at the following concentrations: 50 μg/mL ampicillin (Amp), 12 μg/mL tetracycline (Tet), 30 μg/mL chloramphenicol (Cam), and 50 μg/mL kanamycin (Kan).

Chemicals and Reagents. All of the ultrapure deoxynucleoside triphosphates (dNTPs) were purchased from New England Biolabs. [γ-^{32}P]ATP (6000 μCi/μmol) and ATP were purchased from Amersham and Sigma, respectively. All the oligonucleotides used in this study were made by Keck Molecular Biology Center at Yale University and purified by denaturing polyacrylamide gel electrophoresis (20% acrylamide and 8 M urea).

Generation of I260 Mutants. The method employed to generate I260 mutants followed the Stratagene site-directed mutagenesis protocol where the following primers were used to randomly mutate position I260 of rat *polβ* in the pAraBAD vector background: I260F, 5′ GGA GAA TCG ATA TCA GGT TGN NNC CCA AAG ATC AGT ACT ACT G 3′; and I260R, 5′ CAG TAG TAC TGA TCT TTG GGN NNC AAC CTG ATA TCG ATT CTC C 3′. The PCR conditions were 95 °C for 30 s for 1 cycle followed by 95 °C for 30 s, 55 °C for 1.5 min, and 68 °C for 10 min for 16 cycles.

The following sets of F and R (forward and reverse, respectively) primers were used to generate the H, Q, K, Y, R, N, E, and Stop mutations as position 260 in the pET28a-(+) vector background as described in the Stratagene site-directed mutagenesis protocol: I260HF, 5′ GGA GAA TCG ATA TCA GGT TGC ATC CCA AAG ATC AGT ACT ACT G 3′; I260HR, 5′ C AGT AGT ACT GAT CTT TGG GAT GCA ACC TGA TAT CGA TTC TCC 3′; I260QF, 5′ GGA GAA TCG ATA TCA GGT TGC AGC CCA AAG ATC AGT ACT ACT G 3′; I260QR, 5′ C AGT AGT ACT GAT CTT TGG GCT GCA ACC TGA TAT CGA TTC TCC 3′; I260KF, 5′ GGA GAA TCG ATA TCA GGT TGA AAC CCA AAG ATC AGT ACT ACT G 3′; I260KR, 5′ CAG TAG TAC TGA TCT TTG GGT TTC AAC CTG ATA TCG ATT CTC C 3′; I260YF, 5′ GGA GAA TCG ATA TCA GGT TGT ATC CCA AAG ATC AGT ACT ACT G 3′; I260YR, 5′ CAG TAG TAC TGA TCT TTG GGA TAC AAC CTG ATA TCG ATT CTC C 3′; I260RF, 5′ GGA GAA TCG ATA TCA GGT TCG CGC CCA AAG ATC AGT ACT ACT G 3′; I260RR, 5′ CAG TAG TAC TGA TCT TTG GGG CGC AAC CTG ATA TCG ATT CTC C 3′; I260NF, 5′ CCG CAA TCG ATA TCA GGT TGA ATC CCA AAG ATC AGT ACT ACT G 3′; I260EF, 5′ GGA GAA TCG ATA TCA GGT TGG AAC CAA AAG ATC AGT ACT ACT G 3′; I260ER, 5′ CAG TAG TAC TGA TCT TTG GGT TCC AAC CTG ATA TCG ATT CTC C 3′; I260STOPF, 5′ GGA GAA TCG ATA TCA GGT TGT AAC CCA AAG ATC AGT ACT 3′; I260STOPR, 5′ CAG TAG TAC TGA TCT TTG GGT TAC AAC CTG ATA TCG ATT 3′; I260DF, 5′ GGA GAA TCG ATA TCA GGT TGG ATC CCA AAG ATC AGT ACT 3′; and I260DR, 5′ CAG TAG TAC TGA TCT TTG GGA TCC AAC CTG ATA TCG ATT 3′. The PCR conditions were 95 °C for 30 s for 1 cycle followed by 95 °C for 30 s, 55 °C for 1.5 min, and 68 °C for 12.5 min for 16 cycles.

DNA was isolated (Qiagen Miniprep) and sequenced (Keck Molecular Biology Center at Yale University) using the 432+b primer (5′ GAA CCA CCA TCA GCG AAT TGG 3′) for the pBAD constructs and the T7 forward primer (5′ TAA TAC GAC TCA CTA TA 3′) for the pET constructs.

In Vivo Activity Assays. Mutant plasmids in the pAraBAD vector background were electroporated into SC18-12 and assayed for their ability to complement the *polA* temperature sensitive mutation as previously described (*19*). Strains carrying wild-type or mutant *polβ* were grown at 30 °C overnight in NB Tet Amp and radially streaked onto NA Tet Amp with or without 0.2% arabinose for the rotary streak assay, or dilutions were plated onto plates with and without inducer (0.2% arabinose) for the quantitative complementation assay. Plates were incubated at 30 or 37 °C. For the quantitative complementation assay, colonies were counted from plates incubated at both 30 and 37 °C with and without induction. The percent complementation equaled (the number of colonies at 37 °C with induction)/(the number of colonies at 30 °C) × 100.

Identification of Mutator Mutants in the Trp^+ Reversion Assay. Mutator mutants were identified in a genetic screen as previously described (*20*). Briefly, wild-type or mutant Polβ in the SC18-12 strain background was grown overnight in LBTetAmp with 0.2% arabinose at 37 °C. Dilutions were made in PBS (pH 7) and plated on both ET and Eglu plates, and mutator frequencies were calculated [(number of colonies per milliliter on Eglu)/(number of colonies per milliliter on ET)].

Expression and Purification of Proteins. Wild-type and mutant proteins were expressed from the pET28a(+) vector in the BL21DE strain background. This created N-terminal six-His tag fusion proteins that were purified as previously described (*9*) using a fast liquid chromatography system over a Ni^{2+} affinity column followed by an ion exchange column. Proteins were more than 90% pure as determined with Coomassie Blue-stained SDS−PAGE gels. Concentrations of Polβ proteins were calculated on the basis of an ϵ_{280} of 21 200 M^{-1} cm^{-1} and a molecular mass of 40 kDa for the His-tagged protein or an ϵ_{280} of 10 493 M^{-1} cm^{-1} for the I260Stop mutant with a molecular mass of 29.5 kDa.

In Vitro Activity/Primer Extension Assay. Oligonucleotides CIIU (primer), CIIT (template), and CII45D (downstream oligonucleotide) were used to construct the following 5 bp gapped DNA substrate

5' TTG CGA CTT ATC AAC GCC CAC A AGT TGT CTT CTC AGT CCT 3'

3' AAC GCT GAA TAG TTG CGG GTG TAG TCA TCG ACA GAA GAG TCA GGA 5'

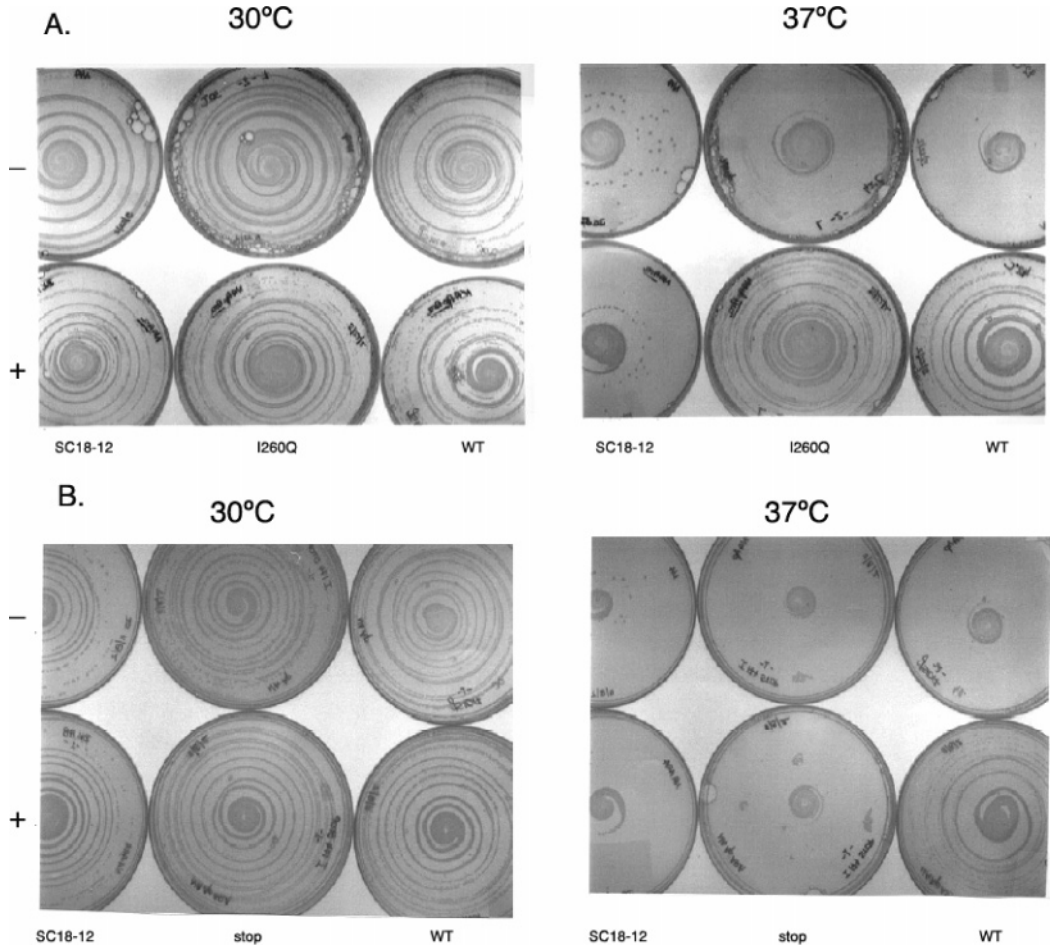

FIGURE 2: Rotary streak assay. SC18-12 cells or SC18-12 cells carrying the plasmid encoding either wild-type Polβ or I260 mutants of Polβ were grown on NA at 30 or 37 °C either with (+) or without induction (−). The rotary streaks were achieved by drawing an inoculating loop slowly across the radius of a spinning plate to create a cell density gradient from the center to the perimeter of the plate. Results typical of a complementing mutant, I260Q, are shown in panel A, where the background SC18-12 strain is on the left, I260Q is in the middle, and the wild type is on the right; the noncomplementing mutant I260stop is shown in panel B with the background SC18-12 strain on the left, I260stop in the middle of the panel, and the wild type on the left.

The primer oligonucleotide was labeled at the 5′ end using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]-ATP. The template and downstream oligonucleotide were 5′ labeled with nonradioactive ATP. Kinased oligonucleotides were purified using a Microspin P-30 column (Bio-Rad) and annealed at a primer:template:downstream oligonucleotide molar ratio of 1:1.2:1.3 in 50 mM Tris (pH 8.0) and 250 mM NaCl. The mixture was incubated sequentially at 95 °C for 5 min, slow-cooled to 50 °C for 30 min, and incubated at 50 °C for 20 min followed by immediate transfer to 4 °C. The quality of the annealed product was monitored on an 18% native polyacrylamide native gel followed by autoradiography as previously described (*17*) to ensure >98% of the oligonucleotides annealed.

A gap filling assay was performed to test the polymerase activity of DNA Polβ mutants under single-turnover conditions where the enzyme:DNA substrate molar ratio is 15:1. In these experiments, a solution containing 50 nM ^{32}P-end-labeled primer−template and 750 nM enzyme (wild-type or mutant Polβ) was preincubated at 37 °C for 1 min in a buffer consisting of 50 mM Tris (pH 8.0), 20 mM NaCl, 2 mM DTT, and 10% glycerol; the reactions were initiated by addition of 50 μM dNTPs and 10 mM $MgCl_2$ in the same buffer and terminated with 0.5 EDTA after a 5 min incubation at 37 °C. The reaction products of primer extension by one to five nucleotides were resolved on a 20% Sequel NE (American Bioanalytical) polyacrylamide gel. The bands were visualized by an Amersham Biosciences Storm 840 PhosphorImager.

In Vitro Mutator Activity. The same 5 bp gapped substrate described in the primer extension assay was used to test the in vitro mutator activity of the mutator mutants identified in the in vivo Trp$^+$ reversion assay. The reactions were performed as described above except pools (containing each dNTP at 50 μM) missing either one or two nucleotides were used in these reactions.

Circular Dichroism. For the wavelength scan measurements, wild-type or mutant enzymes (6, 8, or 10 μM) in 10 mM K_2HPO_4 (pH 8.0) were incubated in a quartz cuvette with a path length of 0.2 cm. The sample was placed in a thermostated block in a circular dichroism spectrophotometer (Aviv model 305Sf). Molar ellipticity (in degrees per square centimeter per decimole) was measured at each wavelength ranging from 260 to 190 nm. Three measurements were taken for each enzyme. For the melting temperature study, ellipticity was measured at 220 nm as a function of temperature over the range of 10−60 °C in 1 °C increments after the sample was equilibrated for 30 s at each temperature. Values were averaged for 15 s, and three measurements were taken for each enzyme. The temperature at which the protein is

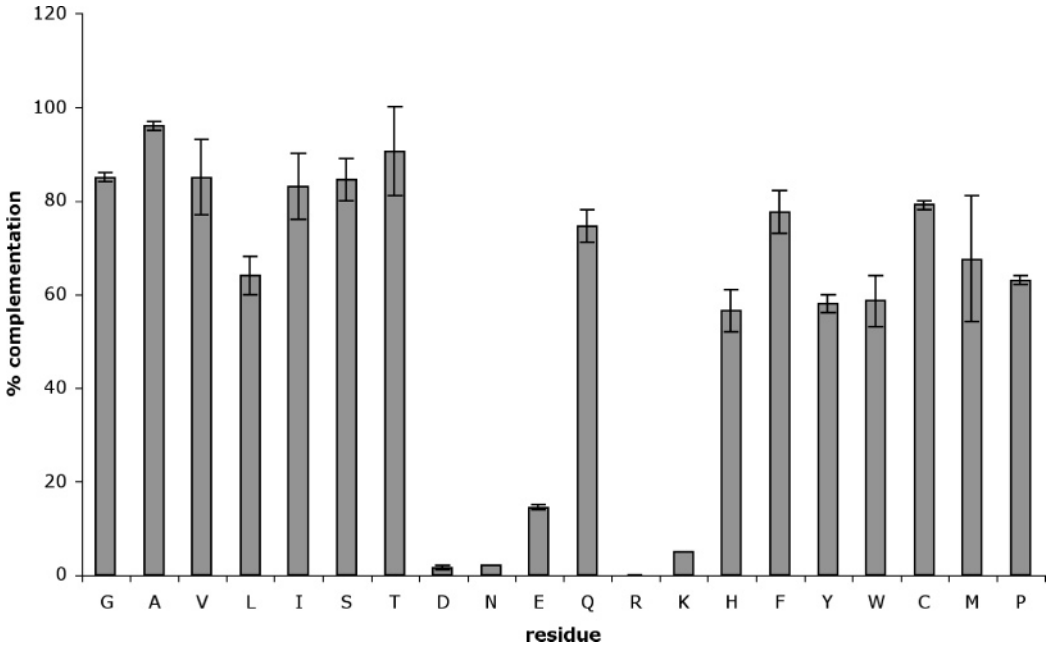

FIGURE 3: I260D, -N, -E, -R, and -K exhibit impaired activity in the in vivo complementation assay. Cultures of SC18-12 carrying the plasmid encoding wild-type Polβ or I260 mutants were diluted in PBS (pH 7.0) and spread on plates with and without arabinose. Plates were grown at either 30 or 37 °C, and colonies were counted to calculate the number of viable cells per milliliter. The percent complementation was calculated by dividing the number of cells at 37 °C with induction by the number of viable cells at 30 °C and multiplied by 100. The bar graph documents the percent complementation (*y*-axis) by all the I260 mutants (*x*-axis). Results are representative of four independent experiments. The standard deviation is represented by error bars.

50% unfolded was calculated by subtracting upper and lower baselines from the denaturation profile.

RESULTS

Lys, Arg, Asp, Asn, and Glu Substitutions at Position 260 of Polβ Yield Enzymes with Impaired Activity. To determine if hinge residue I260 is important for the activity and fidelity of Polβ, we mutated it to each of the other amino acids and the stop codon. We used a genetic screen based upon findings by Witkin and colleagues (*21*) to identify the active I260 variants of Polβ. This screen is based on the ability of mammalian DNA polymerase β to substitute for the polymerization and repair functions of Pol I in *Escherichia coli* (*19*, *22*). The screen is performed in a strain background with a temperature sensitive mutation in *polA*, the gene encoding Pol I. This mutation allows strain growth at 30 °C, but upon switching to the nonpermissive temperature of 37 °C, the bacteria survive only if the Pol I deficiency is complemented by wild-type Polβ or another active variant. For the purpose of this assay, the Polβ variants and wild type are present on an arabinose inducible plasmid to test the dependence of complementation on Polβ induction. The assay is performed by rotary streaking, a qualitative approach based on diluting the culture from the center to the periphery of the plate. As shown in Figure 2A, the SC18-12 strain is able to grow as single colonies at 30 °C but not at 37 °C. However, the SC18-12 strain expressing wild-type Polβ is able to grow at both 30 and 37 °C in the presence of the inducing agent, arabinose. The middle panel of Figure 2A shows that I260Q is also able to grow as single colonies at 37 °C, suggesting it is an active mutant of Polβ. Figure 2B shows an example of a noncomplementing mutant, I260stop, which both in the presence and in the absence of induction fails to grow as single colonies at 37 °C.

The 20 variants that we constructed were tested in this screen, and only I260D, I260N, I260E, I260R, I260K, and I260stop were unable to complement (data not shown). To quantitatively estimate the levels of complementation by the different variants, serial dilutions of logarithmic phase cultures were plated on NA with and without the inducer arabinose and colonies were counted. In Figure 3, we show a bar graph of the quantitative experiments performed with each variant of Polβ which confirm our findings from the qualitative assay in that all variants, except for D, N, E, R, and K substitutions, are able to complement significantly the *polA* deficiency of this strain. Expression of wild-type Polβ (I on the *x*-axis) in the SC18-12 strain background results in approximately 80% complementation of the *polA* defect. This is somewhat higher than we originally reported but using IPTG, instead of arabinose, as an inducing agent (*19*). The majority of the I260 mutants, including I260G, -A, -V, -S, -T, -Q, -F, and -C, exhibit near-wild-type levels of complementation. The I260L, -H, -Y, -W, -M, and -P mutants appear to confer slightly lower levels of complementation than the wild-type enzyme. I260E exhibits ~15% complementation, whereas the D, N, R, and K variants show little or no complementation. Surprisingly, these data suggest that residue 260 of the hinge region of Polβ can tolerate many different amino acid substitutions that still allow the enzyme to remain active.

The Variants of I260 with Impaired in Vivo Complementation Ability Also Exhibit Significantly Reduced Levels of Polymerase Activity in Vitro. We wished to examine if the impairment in complementing the growth defect of the SC18-12 strain was due to a deficiency in polymerase activity. We designed a substrate with a 5 bp gap that was labeled at its 5′ primer terminus, mixed the substrate, wild-type or variant Polβ, and all four dNTPs, and allowed them to react for 5

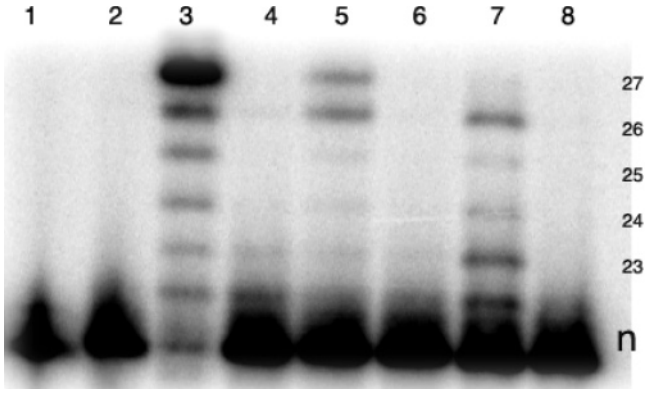

FIGURE 4: In vitro activity primer extension assay. Purified enzymes were incubated with the radiolabeled DNA substrate and all four dNTPs for 5 min at 37 °C as described in Materials and Methods. Products were separated by denaturing gel electrophoresis and visualized using a Phosphorimager. *n* indicates the location of the primer. Lanes are numbered as follows: lane 1, primer control; lane 2, I260stop; lane 3, wild type; lane 4, I260K; lane 5, I260N; lane 6, I260D; lane 7, I260E; and lane 8, I260R. The numbers indicate the length of the extended primer; *n* is the position of the primer.

min. The reactions were quenched, and the products were resolved on a denaturing gel. Given that Polβ is a distributive enzyme, addition of each individual nucleotide to the 22-mer primer should be observed, and thus 22-, 23-, 24-, 25-, 26-, and 27-mers would be expected to be seen for the active enzymes. The wild type was used as an example of an enzyme expected to exhibit polymerase activity. The I260stop mutant was also tested and expected not to be active as indicated from its inability to complement significantly the *polAts* mutation in the in vivo assay. As shown in Figure 4, the wild type fully extends the primer, and exhibits strand displacement synthesis as indicated by the fact that the majority of the product is 27 nucleotides long. I260D, -K, -R, and -stop barely extend the primer as evidenced by the most intense band being 22 nucleotides in length, thus mirroring their in vivo phenotype. Low levels of primer extension were observed with I260N and I260E, which appear to exhibit extremely low levels of complementation in vivo (5 and 15%, respectively). This assay was also performed at 22 and 34 °C to test whether activity was a function of protein stability (data not shown). The results were the same for all the temperatures that were tested, indicating that the enzymes with N, K, R, D, and E substitutions at position 260 have impaired polymerase activity.

The Inactive I260 Mutants Appear To Have Wild-Type Patterns of Folding. We decided to examine if the dramatically reduced complementation ability relative to that of the wild type and the impairment in polymerase activity detected for the D, N, E, R, and K substitutions at position 260 of Polβ were a function of gross changes in the enzyme's folding patterns. For this purpose, a circular dichroism wavelength scan was performed. Figure 5 shows the results of the wavelength scans; in Figure 5A, molar ellipticity is measured as a function of wavelength for 6 μM wild type, I260N, I260R, and I260K. I260N, I260R, and I260K have the same general folding patterns as the wild type with the predominant α-helical fold characteristic of the molar ellipticity pattern observed between 220 and 230 nm. In Figure 5B, a wavelength scan was performed with 8 μM

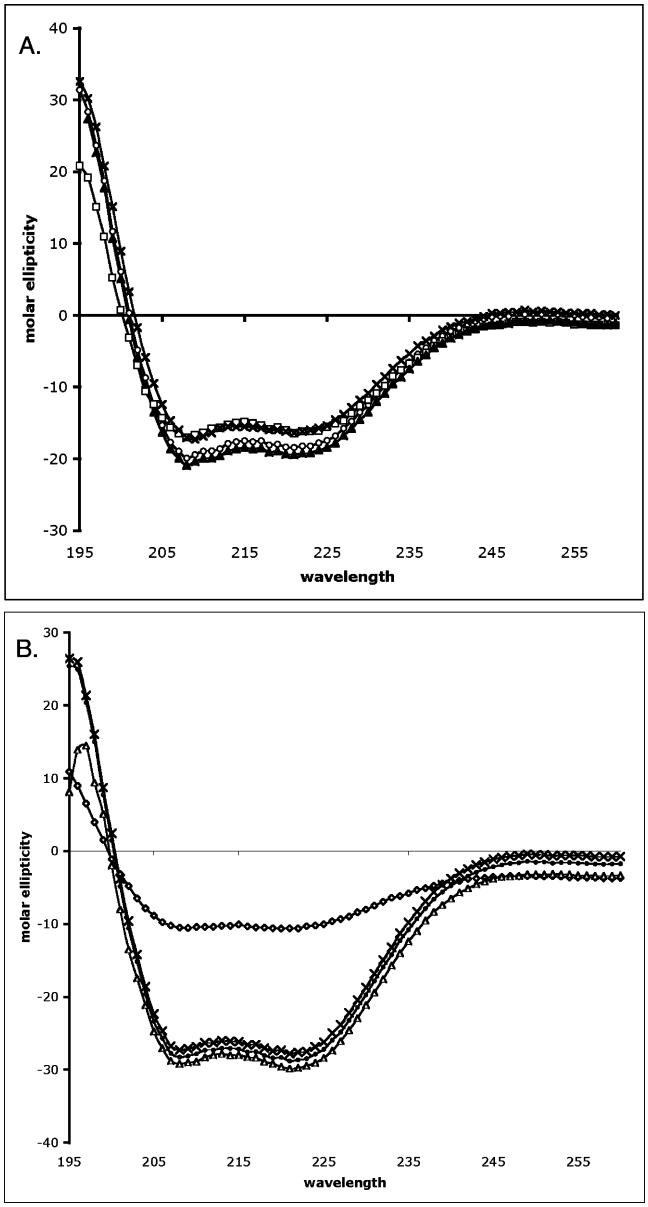

FIGURE 5: Circular dichroism wavelength scan of the inactive I260 mutants. Purified proteins were prepared and scanned in a circular dichroism spectrophotometer as described in Materials and Methods. Molar ellipticity (*y*-axis) in degrees per square centimeter per decimole is plotted vs wavelength (*x*-axis). The data series are as follows: (A) wild type (×), I260R (○), I260N (▲), and I260K (□) and (B) wild type (×), I260E (◇), I260D (△), and I260stop (●).

wild type, I260E, I260D, and I260stop. All of these variants, except for I260stop, were found to exhibit equivalent levels of mostly α-helical folds. I260stop, although appearing to be mostly composed of α-helices, as expected, shows a significantly reduced level of molar ellipticity of -10 as compared to the value of -30 of the wild type and the other two variants. The differences observed between the wild type in panels A and B are a function of the concentration of the enzyme that was used (8 and 6 μM, respectively). In addition, molecular modeling of energy-minimized mutant structures indicated the general folding patterns to be the same as for the wild-type enzyme (data not shown). These data suggest that the impairment seen in the activity of these variants is not due to any major structural changes relative to the wild type.

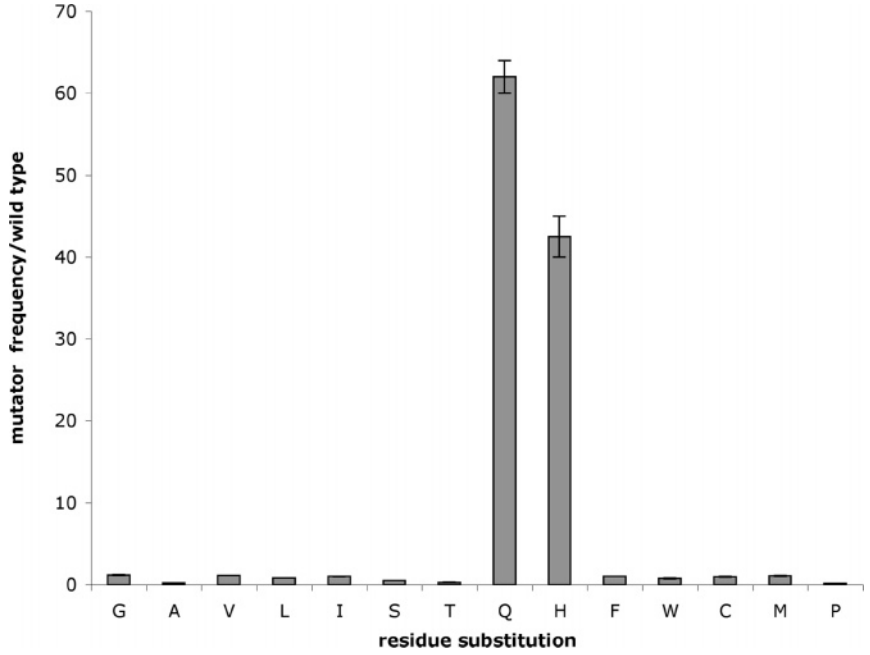

FIGURE 6: Trp$^+$ reversion assay for detecting mutator I260 mutants of Polβ. SC18-12 cells carrying a plasmid encoding wild-type or mutant Polβ were grown as described in Materials and Methods. Overnight cultures were diluted in PBS (pH 7.0) and plated on ET and Eglu plates. Mutation frequency was calculated as (the number of colonies on Eglu)/(the number of colonies on ET). The bar graph was plotted to show the residue substitution on the *x*-axis and the mutation frequency relative to that of the wild type on the *y*-axis. The results are averaged from four independent experiments. The standard deviation of the mean is represented by error bars.

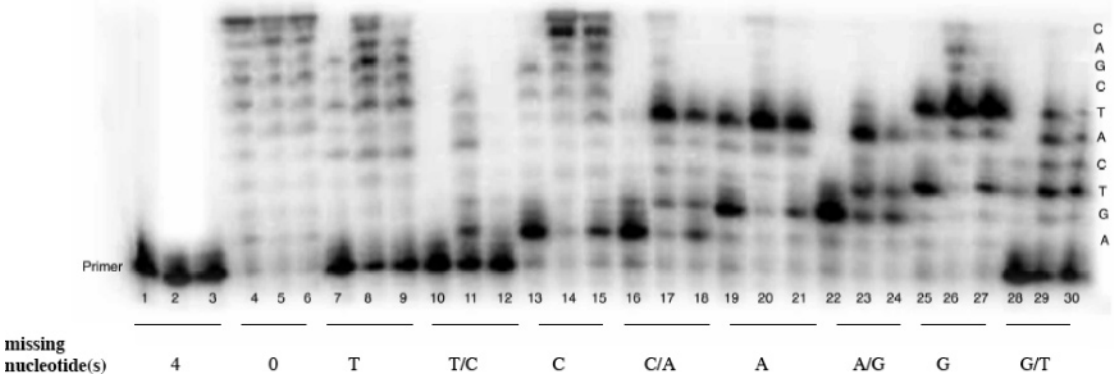

FIGURE 7: In vitro mutator primer extension assay. Purified wild type, I260Q, or I260H (750 nM) was incubated with radiolabeled DNA substrate (50 nM) and either all four dNTPs or a pool of dNTPs missing one or two nucleotides. The reactions were performed as described in Materials and Methods. The products resulting from incorporation of nucleotides into the primer were resolved on a denaturing acrylamide gel and visualized on a Phosphorimager. *n* indicates the position of the primer; the missing nucleotides are indicated at the bottom. The reactions are loaded in sets of three in the following order: wild type, I260H, and I260Q. The wild type is in lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, and 28. I260H is in lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, and 29. I260Q is in lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30. The missing nucleotide(s) is indicated at the bottom. The letters on the side indicate the template nucleotide sequence.

The I260H and I260Q Variants Have a Mutator Phenotype. To determine if any of the active I260 variants display mutator activity, we employed a Trp$^+$ reversion screen (*20*). The *trpE* gene encodes anthranilate synthetase, an essential enzyme in the trypthophan synthesis pathway. The *trpE65* strain background is unable to produce tryptophan due to an ochre mutation in the *trpE* gene and therefore has to be maintained on medium containing tryptophan. A Trp$^+$ reversion phenotype can be detected as a result of five of six possible base substitution errors occurring from incorrect insertion of dNTP into the *trpE* gene or into the anticodon loop of suppressor tRNAs by an error prone variant polymerase. As shown in Figure 6, the reversion frequencies relative to that of the wild type are similar for all the active mutants except for the Gln and His variants. Interestingly, the Gln and His variants exhibit ~60- and ~45-fold increases in mutator frequency, respectively, relative to that of the wild type, suggesting that they are mutator mutants.

I260H and I260Q Exhibit Mutator Activity in Vitro. To test whether the in vivo mutator activity of I260H and I260Q is an inherent characteristic of these enzymes, we developed an in vitro gap filling assay. The purified enzymes were mixed with the DNA substrate and either all four or a combination of two or three nucleotides as described in Materials and Methods. In the case of all four nucleotides, in vitro polymerase activity was tested for both I260Q and I260H, showing both enzymes to be active (Figure 7, lanes 5 and 6) and their activity to be nearly equivalent to that of the wild-type enzyme (lane 4). In addition to being able to fill the gap, the two mutants are able to perform strand displacement synthesis as is the wild-type enzyme, as seen by their ability to generate products more than 27 nucleotides

in length. Next, we used only three nucleotides to determine if these enzymes were able to misinsert opposite the base where the missing nucleotide was to be incorporated. In the case of I260H and I260Q, misinsertions were identified (lanes 8 and 9, 14 and 15, 20 and 21, and 26 and 27 where I260H is loaded into the even-numbered lanes and I260Q into the odd ones), whereas the wild type was not able to misinsert, stopping one nucleotide before the point where the missing nucleotide was to be inserted (lanes 7, 13, 19, and 25 are the wild type, each corresponding to the equivalent pair of nucleotide pool lanes listed above). For instance, lanes 13−15 illustrate the ability to misincorporate opposite template G. In the absence of dCTP from the nucleotide pool, wild-type Polβ exhibits primer extension to 23 nucleotides and stops exactly one nucleotide before the position where C should be inserted opposite G (lane 13), and both I260Q and I260H are able to continue polymerizing past the point of the missing C, extending the primer to ~28 nucleotides (lanes 14 and 15). Because of its intrinsically low fidelity, the wild-type enzyme also exhibits a level of mutator activity as seen in lanes 19 and 25 (when dATP and dGTP are missing from the nucleotide pool, respectively), but these levels of misincorporation are significantly lower than those for I260H or I260Q (compare lane 19 to lanes 20 and 21 and lane 25 to lanes 26 and 27). These data indicate that both I260Q and I260H are able to misincorporate nucleotides, confirming their in vivo mutator activity.

Mutator Mutants Are Properly Folded but Less Stable than the Wild Type. Circular dichroism revealed no major differences in the general folding patterns of these two mutants as compared to the wild-type enzyme with the α-helical structures still being predominant and folded to the same level as measured by molar ellipticity (Figure 8A). A temperature melt from 0 to 60 °C allowed us to plot the melting curves for the wild type and the two variants and revealed the stability of both I260Q and I260H to be lower than that of the wild type. As determined from Figure 8B, the wild type's melting temperature is 41.5 °C while the T_m for I260H is 40 °C and for I260Q 37 °C. These data suggest that the I260H and I260Q mutator mutants are slightly less stable than the wild type.

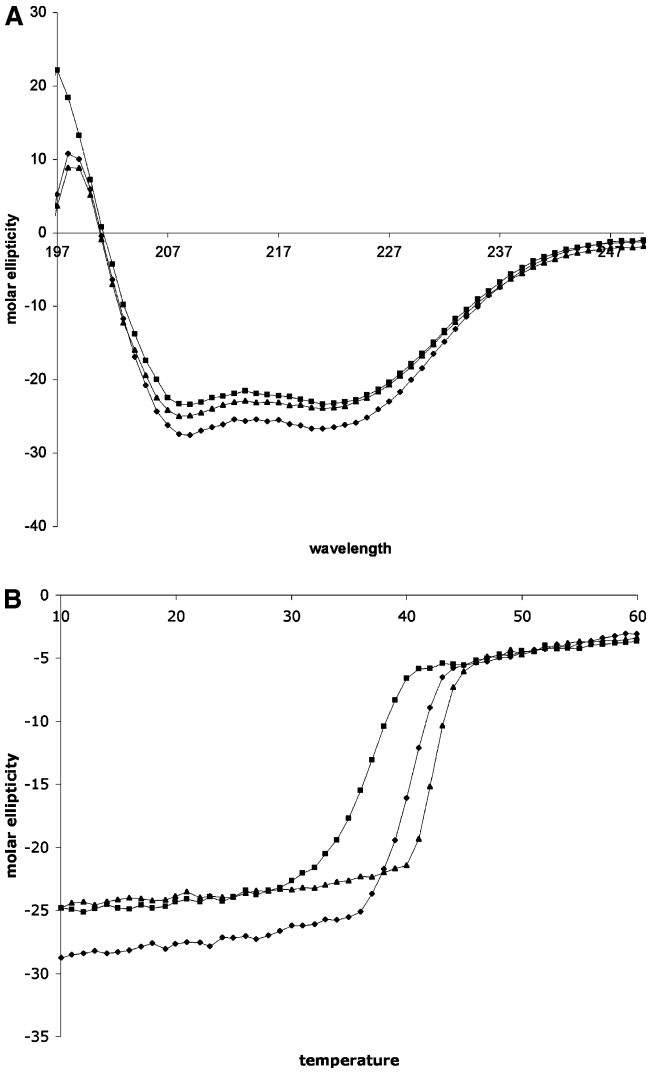

FIGURE 8: (A) Examination of general folding patterns of I260Q and I260H. Overall folding patterns of I260Q and I260H were compared to that of the wild type by a circular dichroism wavelength scan as described for the inactive mutants of I260. Molar ellipticity (*y*-axis) is plotted vs the wavelengths at which it was measured (*x*-axis). (B) Melting temperature studies of I260Q and I260H. The studies were performed at 22 nm as described in Materials and Methods. The temperature is plotted vs molar ellipticity. The melting temperature (T_m) was calculated after the upper and lower baselines were subtracted from the denaturation profile: wild type (■), I260H (▲), and I260Q (◆).

DISCUSSION

In the work presented here, we have mutated hinge residue isoleucine 260 of DNA polymerase β to all other amino acids and to the stop codon, creating a total of 20 mutants. Surprisingly, we found this residue in the hinge of the enzyme to be permissive for mutations that conserve the nonpolar nature of the residue, rather than residue size, allowing the enzyme's activity to be maintained as shown in our in vivo complementation assay. We also show, in our Trp^+ reversion assay, that the variants that conserve the hydrophobic nature of the residue, in addition to being active, do not have mutator activity. In contrast, when charged residues or highly hydrophilic polar residues were substituted into position 260, they gave rise to enzymes with impaired activity or active enzymes with a mutator phenotype. These data show that residue I260 of the hinge must maintain its hydrophobic character for Polβ to be active and accurate. Our results strongly suggest that the hinge is crucial for the activity and fidelity of this polymerase.

Subtle Changes in the Hinge Environment Compromise Fidelity. Variants with impaired activity or low fidelity maintain the general folding patterns of the wild-type enzyme, indicating that it is not an overall change in the folding patterns of the protein that leads to altered activity or fidelity. We suggest that subtle changes in the local environment of the hinge change Polβ's activity and accuracy. Molecular modeling of the energy-minimized structures of I260Q and the wild-type enzyme revealed that the DNA substrate with its templating residue is positioned several planes directly above reside 260 as depicted in Figure 1C. If one could imagine the effects of the Polβ hinge to be propagated in the shape of a cone, the tip of the cone would correspond to residue 260, while the large flat surface of the cone would underlie the DNA substrate in the open conformation of the enzyme. The position of the DNA substrate relative to residue 260 is shown in Figure 1C. We

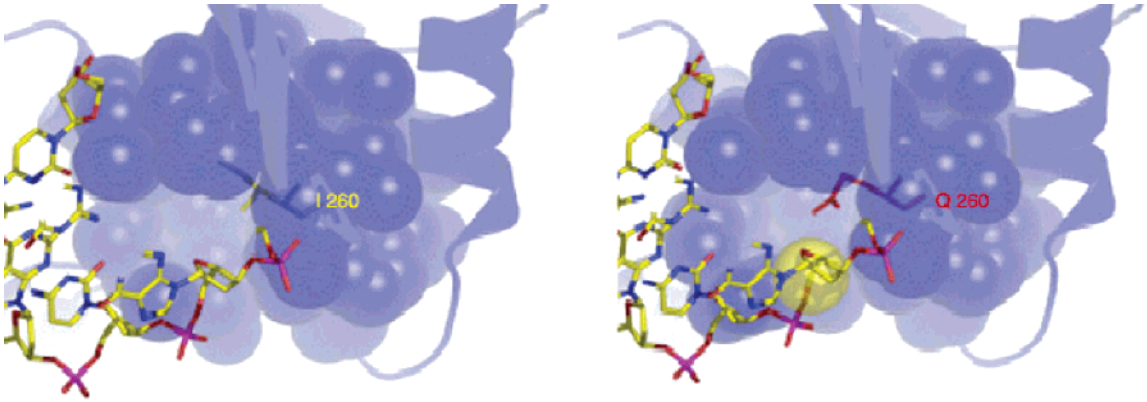

FIGURE 9: Difference in DNA positioning between the wild type and I260Q. This figure was generated using PyMol to visualize Swiss-Model energy-minimized structures of Polβ from Protein Data Bank entry 1bpx. The spheres denote an area of 8 Å around residue 260. The wild type is at the left where the area outlined by spheres does not include the DNA substrate (cyan spheres), whereas the I260 variant is at the right where the same volume partially encompasses the DNA substrate (yellow sphere).

postulate that any changes at the tip of the cone, the actual location of residue 260, could lead to subtle local changes that domino out toward the surface of the cone and as such are reflected in the plane that accommodates the DNA substrate. Molecular modeling of the I260Q variant revealed a difference in the position of the DNA substrate in the open conformation of the enzyme. In this variant, the DNA substrate appears to have shifted from the surface of the cone further into the body of the hinge (Figure 9). This could lead to an altered DNA structure at the time of the enzyme being in the process of binding the dNTP substrate. Because the DNA forms part of the dNTP binding pocket (*23*), altered DNA positioning could lead to accommodation of the incorrect dNTP substrate. It is also worth noting that our modeling studies did not reveal any differences in the positioning of arginine 283 or other amino acid residues involved in setting up the transition state and activating the enzyme between the wild type and the mutants that were tested. This might indicate that there are no significant differences in the transition state or activation process between the wild type and the I260 variants but that the empirically observed impairments in activity and fidelity are due to altered positioning of the DNA substrate.

We did not detect a difference in the position of the DNA substrate with the other activity- or fidelity-impaired enzymes in our modeling studies. However, we postulate that such differences in the position of the DNA substrate might be present in the other inactive or inaccurate variants of Polβ but are too subtle to be seen in our simulated modeling system. We found that the general volume of the area around the hinge changed in the variants with lowered polymerase activity but not in the active ones we examined (data not shown). It is also worth noting that the effects seen on the volume of the area around the hinge in these variants were not due to any major changes in any one residue comprising the area, but rather resulted from a cumulative effect of numerous minor changes (of 0.2−0.3 Å each).

The simplest explanation for the phenotypic effects observed with these other mutants is that such minimal structural changes are sufficient to alter the active site geometry of the enzyme in a very subtle way. This could result in altered positioning of the template, primer, and/or dNTP substrate and could affect the ability of the enzyme variants to efficiently or acurately catalyze phosphodiester bond formation. For the inactive mutants, the active pocket size or shape may change so that the transition state cannot be assumed or stabilized. For I260H, the size or geometry of the active pocket could be permissive for the accommodation of the incorrect dNTP substrate, as suggested for I260Q.

Residue Size Does Not Influence Activity or Fidelity. We found that the size of the residue substituted at position 260 does not affect the activity or fidelity of the enzyme. We expected to find an effect on the enzyme's activity and/or fidelity depending on the size, or more precisely wan der Waals volume of the reside at position 260. This hypothesis was borne out by the fact that this residue is positioned such that any volume-changing effects seen locally (due to its location at the tip of the cone) would be amplified in the surface of the cone where the DNA substrate is positioned. However, we found Asn to be inactive and Leu and Val to have wild-type levels of activity, although they are all close to the size of the wild-type residue, isoleucine. Therefore, we conclude that the positive or polar residues alter the chemical enviroment of the hinge and affect the stacking of the residues around residue 260 which is then felt in the planes above residue 260, including the plane of the DNA substrate.

Changing the chemical nature of the residue alone has two different phenotypic effects: some affecting the activity of the enzyme and others not seemingly affecting activity within the confines of the assay we use, but severely impairing the accuracy of incorporation. The effects we see by changing a single amino acid residue in an active site distant region of the enzyme point to the critical role of the residues distant from the active site.

Understanding the precise and carefully evolved mechanism that functions in favor of enzyme fidelity is crucial to understanding the impairments and deficiencies in the processes in which these enzymes participate. Several Polβ variants have been associated with various types of tumors, one of them a hinge variant (S. Dalal et al., manuscript in preparation). Given Polβ's participation in base excision repair and its implication in other cellular processes (*12*, *24*−*26*), it is important to understand precisely how deficiencies in this enzyme affect the processes in which it participates. Further insights into the mechanistic details of catalysis could prove to be of essence in designing treatment strategies.

ACKNOWLEDGMENT

We thank Steve Kaiser for his help with molecular modeling. We also thank Agnes Keh for technical support.

REFERENCES

1. Ochs, K., Sobol, R. W., Wilson, S. H., and Kaina, B. (1999) Cells deficient in DNA polymerase β are hypersensitive to alkylating agent-induced apoptosis and chromosomal breakage, *Cancer Res. 59*, 1544−1551.
2. Horton, J. K., Baker, A., Berg, B. J., Sobol, R. W., and Wilson, S. H. (2002) Involvement of DNA polymerase β in protection against the cytotoxicity of oxidative DNA damage, *DNA Repair 1*, 317−333.
3. Dianov, G., Price, A., and Lindahl, T. (1992) Generation of single-nucleotide repair patches following excision of uracil residues from DNA, *Mol. Cell. Biol. 12*, 1605−1612.
4. McCullough, A. K., Dodson, M. L., and Lloyd, R. S. (1999) Initiation of base excision repair: Glycosylase mechanisms and structures, *Annu. Rev. Biochem. 68*, 255−285.
5. Raffoul, J. J., Cabelof, D. C., Nakamura, J., Meira, L. B., Friedberg, E. C., and Heydari, A. R. (2004) Apurinic/apyrimidinic endonuclease (APE/REF-1) haploinsufficient mice display tissue-specific differences in DNA polymerase β-dependent base excision repair, *J. Biol. Chem. 279*, 18425−18433.
6. Wilson, T. M., Rivkees, S. A., Deutsch, W. A., and Kelley, M. R. (1996) Differential expression of the apurinic/apyrimidinic endonuclease (APE/ref-1) multifunctional DNA base excision repair gene during fetal development and in adult rat brain and testis, *Mutat. Res. 362*, 237−248.
7. Matsumoto, Y., and Kim, K. (1995) Excision of deoxyribose phosphate residues by DNA polymerase β during DNA repair, *Science 269*, 699−702.
8. Prasad, R., Singhal, R. K., Srivastava, D. K., Molina, J. T., Tomkinson, A. E., and Wilson, S. H. (1996) Specific interaction of DNA polymerase β and DNA ligase I in a multiprotein base excision repair complex from bovine testis, *J. Biol. Chem. 271*, 16000−16007.
9. Kosa, J. L., and Sweasy, J. B. (1999) The E249K mutator mutant of DNA polymerase β extends mispaired termini, *J. Biol. Chem. 274*, 35866−35872.
10. Shah, A. M., Conn, D. A., Li, S. X., Capaldi, A., Jager, J., and Sweasy, J. B. (2001) A DNA polymerase β mutator mutant with reduced nucleotide discrimination and increased protein stability, *Biochemistry 40*, 11372−11381.
11. Dalal, S., Kosa, J. L., and Sweasy, J. B. (2004) The D246V mutant of DNA polymerase β misincorporates nucleotides: Evidence for a role for the flexible loop in DNA positioning within the active site, *J. Biol. Chem. 279*, 577−584.
12. Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H., and Kraut, J. (1994) Crystal structure of rat DNA polymerase β: Evidence for a common polymerase mechanism, *Science 264*, 1930−1935.
13. Shah, A. M., Li, S. X., Anderson, K. S., and Sweasy, J. B. (2001) Y265H mutator mutant of DNA polymerase β. Proper teometric alignment is critical for fidelity, *J. Biol. Chem. 276*, 10824−10831.
14. Werneburg, B. G., Ahn, J., Zhong, X., Hondal, R. J., Kraynov, V. S., and Tsai, M. D. (1996) DNA polymerase β: Pre-steady-state kinetic analysis and roles of arginine-283 in catalysis and fidelity, *Biochemistry 35*, 7041−7050.
15. Opresko, P. L., Sweasy, J. B., and Eckert, K. A. (1998) The mutator form of polymerase β with amino acid substitution at tyrosine 265 in the hinge region displays an increase in both base substitution and frame shift errors, *Biochemistry 37*, 2111−2119.
16. Clairmont, C. A., Narayanan, L., Sun, K. W., Glazer, P. M., and Sweasy, J. B. (1999) The Tyr-265-to-Cys mutator mutant of DNA polymerase β induces a mutator phenotype in mouse LN12 cells, *Proc. Natl. Acad. Sci. U.S.A. 96*, 9580−9585.
17. Shah, A. M., Maitra, M., and Sweasy, J. B. (2003) Variants of DNA polymerase β extend mispaired DNA due to increased affinity for nucleotide substrate, *Biochemistry 42*, 10709−10717.
18. Li, S. X., Vaccaro, J. A., and Sweasy, J. B. (1999) Involvement of phenylalanine 272 of DNA polymerase β in discriminating between correct and incorrect deoxynucleoside triphosphates, *Biochemistry 38*, 4800−4808.
19. Sweasy, J. B., and Loeb, L. A. (1992) Mammalian DNA polymerase β can substitute for DNA polymerase I during DNA replication in *Escherichia coli*, *J. Biol. Chem. 267*, 1407−1410.
20. Washington, S. L., Yoon, M. S., Chagovetz, A. M., Li, S. X., Clairmont, C. A., Preston, B. D., Eckert, K. A., and Sweasy, J. B. (1997) A genetic system to identify DNA polymerase β mutator mutants, *Proc. Natl. Acad. Sci. U.S.A. 94*, 1321−1326.
21. Witkin, E. M., Roegner-Maniscalco, V., Sweasy, J. B., and McCall, J. O. (1987) Recovery from ultraviolet light-induced inhibition of DNA synthesis requires umuDC gene products in recA718 mutant strains but not in recA+ strains of *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 84*, 6805−6809.
22. Sweasy, J. B., Chen, M., and Loeb, L. A. (1995) DNA polymerase β can substitute for DNA polymerase I in the initiation of plasmid DNA replication, *J. Bacteriol. 177*, 2923−2925.
23. Sweasy, J. B. (2003) Fidelity mechanisms of DNA polymerase β, *Prog. Nucleic Acid Res. Mol. Biol. 73*, 137−169.
24. Sobol, R. W., Horton, J. K., Kuhn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K., and Wilson, S. H. (1996) Requirement of mammalian DNA polymerase β in base-excision repair, *Nature 379*, 183−186.
25. Dianov, G. L., Prasad, R., Wilson, S. H., and Bohr, V. A. (1999) Role of DNA polymerase β in the excision step of long patch mammalian base excision repair, *J. Biol. Chem. 274*, 13741−13743.
26. Plug, A. W., Clairmont, C. A., Sapi, E., Ashley, T., and Sweasy, J. B. (1997) Evidence for a role for DNA polymerase β in mammalian meiosis, *Proc. Natl. Acad. Sci. U.S.A. 94*, 1327−1331.

BI047956X