The D246V Mutant of DNA Polymerase β Misincorporates Nucleotides

EVIDENCE FOR A ROLE FOR THE FLEXIBLE LOOP IN DNA POSITIONING WITHIN THE ACTIVE SITE*

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DNA polymerase β, a member of the X family of DNA polymerases, is known to be involved in base excision repair. A key to determining the biochemical properties of this DNA polymerase is structure-function studies of site-specific mutants that result in substitution of particular amino acids at critical sites. In a previous genetic screen, we identified three specific amino acids at critical sites. In this work, we perform an extensive kinetic analysis to investigate the role of the D246V mutant on polymerase fidelity. We find that D246V misincorporates T opposite template bases G and C. The mechanistic basis of misincorporation appears to be altered DNA positioning within the active site. We provide evidence that the fidelity of D246V is greatly affected by the base that is 5′ of the templating base. We propose that the Asp residue at position 246 helps to maintain the proper positioning of the DNA within the polymerase active site and maintains the fidelity of polymerase β. Altogether, the results suggest that the flexible loop domain of polymerase β plays a major role in its fidelity.

A healthy normal cell can be converted to a cancerous cell by the accumulation of mutations (1). We have a limited understanding of how mutations occur in normal cells and how these mutations result in cancer. The base excision repair (BER)1 system repairs 10,000 adducts per cell per day that could otherwise result in mutations (2). BER removes modified bases generated by ionizing radiation, alkylating agents, and endogenous hydrolytic and oxidative processes (reviewed by Ref. 3). Evidence for a role for DNA pol β in base excision repair in mouse cells was obtained by Sobol and colleagues (4), who showed that deletion of pol β results in sensitivity to alkylating damage. BER is initiated by DNA glycosylases, leaving a non-instructive apurinic/apyrimidinic site with mutagenic potential. After incision into the sugar-phosphate DNA backbone by an apurinic/apyrimidinic endonuclease, the resulting 5′-deoxyribose residue is removed by the 5′-deoxyribose phosphatase activity of pol β. pol β also fills the resulting gap in the DNA, and ligase I or III seals the nick (5). The importance of BER in maintaining genomic integrity and reducing cancer susceptibility is evidenced by the fact that mutations and/or different levels of expression of pol β have been observed in many colon and lung tumors and esophageal cancer (6–10). pol β also has a role in meiosis (11).

DNA polymerases differentiate between correct and incorrect substrates from a pool of structurally similar dNTPs and thus maintain the genome of all organisms by accurate DNA synthesis. To begin to understand the origins of this selectivity, it has been important to study the kinetics, thermodynamics, and structure of duplex DNA, of polymerase enzymes, and of their complexes in well defined systems. Although numerous questions remain to be answered, one of the basic questions is how the polymerase structure governs its fidelity. The lack of proofreading activity and relatively small structure (39 kDa) of pol β makes it a most interesting model to understand the fidelity of DNA polymerases. The structure of pol β complexed with single nucleotide gapped DNA, the physiological substrate of pol β, and a ternary complex containing the protein, gapped DNA, and dNTP has been solved (12, 13). The structure of polymerase β shows that like other polymerases, it catalyzes the nucleotidyl transfer reaction by the two metal-ion mechanism (14). In vitro studies show that depending upon the size of the gap and the presence of a 5′-phosphate, pol β can be somewhat processive or distributive (15, 16) and that it has higher fidelity on a single nucleotide gapped DNA (17).

3-Azido-2′,3′-dideoxythymidine 5′-triphosphate (AZT), a thymidine analog, is used as a drug for treating AIDS. It has been reported that overexpression of pol β sensitizes mammalian cells to AZT (18, 19). In vitro, purified DNA pol β incorporates AZT into DNA, causing chain termination (20). AZT is quite similar in structure to thymidine, except that an azido group replaces its 3′-hydroxyl group of the sugar. To obtain insight into how pol β differentiates between AZT and normal dNTPs, an in vivo selection method was developed in our laboratory to identify AZT-resistant mutants of polymerase β (21). In this system, pol β substitutes for DNA polymerase I of Escherichia coli in DNA replication at non-permissive temperature. Mutants were selected from a randomly mutagenized library of pol β clones for their ability to grow on AZT in the recA718polA12 strain at nonpermissive temperatures (21). Several mutants were identified by this method, and among these three independent alterations of pol β, E249K, D246V, and R253M were identified. These alterations reside in a segment of the polypeptide that appears in the crystal structures

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as a disordered or solvent-exposed loop. The presence of three independent mutations in this loop implies that it plays an important role in substrate discrimination. Further characterization of the E249K mutant showed that Glu-249 functions to prevent the extension of a mispaired primer terminus (22). Here we report on the infidelity of the D246V mutant. We show that D246V appears to introduce mutations by altered positioning of DNA within its active site. Our study highlights the importance of the loop domain for the fidelity of pol β.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—The strain BL21 DE3 was used for protein expression and has genotype F-ompT hsdSB (r6K mcrA) gal dcm (DE3).

Chemicals and Reagents—Ultrapure deoxynucleoside triphosphates, ATP, and [γ-32P]ATP were purchased from New England Biolabs, Sigma, and Amersham Biosciences, respectively. Oligonucleotides were synthesized by the Keck Molecular Biology Laboratory at Yale University and purified by denaturing PAGE.

Purification of β-WT and Mutant D246V—The cDNAs of WT D246V were subcloned into the pET28a vector (Novagen) to generate a fusion protein containing six histidine residues attached to the amino terminus. These fusion proteins were expressed and purified as described previously (21), using a fast protein liquid chromatography system. Proteins were greater than 90% homogeneous based on a Coomassie Blue-stained SDS-PAGE gel. Concentrations of pol β proteins were based on ε280 = 21,200 M−1 cm−1 and a molecular mass of 40 kDa for His-tagged pol β.

DNA Substrate Preparation for Kinetic Study—A single base pair gapped DNA substrate and a 6-bp gapped DNA substrate with a 5’-phosphate on the downstream oligonucleotide were used for misincorporation and mispair extension assays, respectively. The sequences of the DNA substrates are shown in Fig. 1. The primer oligonucleotide was labeled at the 5’ end by using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP. Other oligonucleotides were 5’ end-labeled with non-radioactive ATP. Kinased oligonucleotides were purified using a Microspin P-30 column (Bio-Rad) and annealed at a primer/template/dNTP molar ratio of 1:1:2:1 in 50 mM Tris, pH 8.0, 250 mM NaCl. The mixture was incubated sequentially at 95 °C for 5 min, slow-cooled to 50 °C for 30 min and 50 °C for 20 min, and then immediately transferred to ice. The quality of annealing was monitored immediately after mixing. The radiolabeled gapped DNA (300 nM 45X-22-22) was in 3-fold excess relative to pol β (100 nM). These reactions were assayed as burst experiments (24). The burst experiment was performed at saturating concentrations of dNTP while minimizing any substrate inhibition, which may occur with excess dNTP. Reactions were initiated by rapid mixing of the pol β-DNA and Mg-dNTP solutions. At selected time intervals, the reactions were quenched with 0.3 M EDTA. The reaction products were separated and quantitated as described below.

Active Site Titration—The titration was performed by preincubating a fixed concentration of pol β protein with increasing concentrations of the gapped DNA substrate (45A-U22-D22). This mixture was then reacted for 0.3 s with the correct dNTP substrate, dTTP. The selected time interval allowed adequate time to reach maximum amplitude with minimal contribution of multiple catalytic turnovers. This method provides a measurement of the amount of active E-DNA complex present and allows us to obtain the equilibrium dissociation constant, Kd, for DNA.

Single Turnover Misincorporation Assays—To elucidate the relative ability of the D246V 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, Kd, and the maximum rate of polymerization, hmax, for correct and incorrect dNTPs for each enzyme. For single turnover condition assays, reactions were conducted in the same buffer described above for presteady-state kinetic protocols. The kinetics of correct dNTP incorporation were determined under single turnover conditions using the KinTek apparatus (25) thermostated at 37 °C. Single turnover kinetic experiments were performed under conditions where the enzyme concentration greatly exceeds the Kd 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 D246V. These conditions allow binding of greater than 90% of the DNA substrate by pol β and thereby measure the rate of a single catalytic turnover of the enzyme. In these experiments, a solution containing 50 nM 32P-end-labeled primer-template (45X-22-22) and 750 nM enzyme (both polβ-WT and D246V) were used with varying concentrations of

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D246V Mutant of pol β

D246V typically, experiments were carried out by loading 15 μL of the pol β primer-template complex in buffer in one sample loop and 15 μL of a single dNTP in the second sample loop. Reactions were initiated by rapid mixing of the two reactant solutions and were quenched at various times with 0.5 M EDTA. For correct incorporation reactions, substrate concentrations were typically 0–200 μM and reaction times were 0–48 s. The kinetics of misincorporation were determined manually under the above single turnover conditions. Reactions were performed by preincubating 50 nM enzyme with 50 nM 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 M EDTA. For incorrect incorporations, substrate concentrations were typically 0–1 μM, 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% Sequel 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.

**Single Turnover Mispair Extension Assays**—These assays were performed as described above, in single turnover conditions, except the primer-template contained a mispaired terminus (Fig. 1) and the substrate was 45S-U22-D17 instead of 1-nucleotide gapped DNA. The rate of insertion of the next correct nucleotide was measured. Data obtained from kinetic assays were analyzed by using the Kaleidagraph program (Synergy software). The kinetic parameters determined are shown in Scheme 1. Data from the burst experiments were fit to the burst equation: [product] = A (1 − exp(−kobs dt)) + kobs dt. Data from active site titrations were fit to the quadratic equation: [E-DNA] = 0.5Kd[DNA] + [E] + [D] − [0.5KdDNA + [E] + [D]]2 − (2 × [E][D][D]), where [E-DNA] is the concentration of the pol β-DNA complex, [E] is the initial enzyme concentration, [D] is the initial concentration of the gapped DNA substrate, and Kd[DNA] is the dissociation constant of the pol β-DNA complex. Single turnover kinetic data were fit to the single exponential equation: [product] = A (1 − exp(−kobs dt)), where A is the amplitude; t is the time; and kobs is the observed rate constant. Observed rate constants were then plotted against [dNTP], and the data were fit to the burst equation: [product] = kobs [dNTP]t, where kobs is the maximum rate of polymerization, and Kt is the equilibrium dissociation constant for dNTP. Fidelity values were calculated using the relationship: fidelity = (kobs−kpol)/(kobs−kpol)(Kt−kpol), where c and i represent the correct and incorrect dNTPs, respectively.

**RESULTS**

**D246V Shows a Rapid Burst of Product Formation**—D246V was isolated previously as being resistant to AZT (21). Kosa and Sweasy (21) demonstrated that there is a 10-fold reduction in the catalytic efficiency of AZT-TP incorporation by D246V. They also provided evidence that D246V was a mutator mutant of pol β using the missing base primer extension assay (22). In this previous study, results with the specific missing base primer extension assay showed that D246V did not appear to have inherent mutator activity as strong as E249K, and our results with D246V were not always consistent in this assay. However, in the previous assay we employed a recessed DNA substrate, and in recent experiments we have shown that D246V appears to have mutator activity in a missing base primer extension assay with a five-nucleotide gap. Therefore, we decided to use kinetic analysis to fully characterize this mutant.

Our goal in this study was to characterize the mechanism of the mutator activity of D246V. A pre-steady-state burst experiment of purified WT and mutant D246V was performed according to the conditions described under the “Experimental Procedures.” In Fig. 2A we demonstrate that the incorporation of dTTP opposite A by D246V at 37 °C occurs via an initial fast phase (kobs = 6.4 s⁻¹) followed by a slower linear phase (KGS = 0.274 s⁻¹). The observed rate for WT, as shown in Fig. 2B, is 10 s⁻¹, which is similar to that obtained by us previously and by others (34, 35). The biphasic nature of the burst indicates that rate-limiting step of the catalytic cycle occurs after chemistry, similar to WT.

**D246V Misincorporates dTTP Opposite Template C and G**—To better understand the infidelity of the AZT-resistant D246V mutant, we performed single turnover reactions using the 45S-U22-D22 gapped DNA substrate. This allowed us to quantify the relative rate of nucleotide mis-incorporation opposite different templating bases as described under “Experimental Procedures.” Fig. 3 shows an example of a plot of misincorporation of dCTP opposite template C. This enabled us to obtain the maximum rate of polymerization, kpol and the Kt for WT and D246V. The kpol and Kt rate constants were used to calculate the fidelities for WT and D246V. As depicted in Table I, the fidelity of D246V is decreased compared with WT for all misincorporations with the exception of insertion of dATP opposite C. However, the most significant differences between WT and D246V are in the fidelities of misinsertion of dTTP opposite C and G and misincorporation of dCTP opposite template C. D246V has a 23- and 6.4-fold decreased fidelity for misinsertion of dTTP opposite templates C and G, respectively, when compared with WT. Moreover, there is a 10-fold decrease in the fidelity of D246V compared with WT in the case of dCTP misinsertion opposite template T. In these cases, there is little discrimination of correct and incorrect nucleotides during ground state binding by D246V (Kt). The least significant differences between WT and D246V are for misincorporation of dNTPs opposite template A and for misincorporation of dCTP opposite C and dTTP opposite T. However, the kinetic basis of discrimination for incorporation of dNTPs opposite template A and dCTP opposite C appears to have changed for D246V. D246V does not discriminate as well as WT against misincorporation during ground state binding (Kt) but increases its discrimination against misincorporation of dNTPs opposite template A and dCTP opposite C at the level of kpol when compared with WT. The kinetic basis for nucleotide discrimination of dTTP opposite T is the same for WT and D246V.

**Misincorporation Occurs by Altered DNA Positioning**—We noticed that in our DNA substrate the base 5’ to the templating base is G. Misincorporation of dCTP opposite T by D246V could be explained by a repositioning of DNA within the active site such that 5’G becomes the templating base. To test this hypothesis, we constructed a different substrate, 45TTT-U22-D22, in which the base 5’ to the templating base is T. We used single turnover kinetics to determine whether D246V was able to misincorporate dCTP opposite T with this substrate. As shown in Table II, we observe only a 3-fold decrease in the fidelity of D246V compared with WT for insertion of dCTP opposite T. This suggests that misincorporation of dCTP opposite template T is affected by the base 5’ to the templating base.

The fidelity of misincorporation of dCTP by WT opposite template T when T is 5’ to the templating base (22,000) is similar to that when G is 5’ to the templating base (16,400). However, the kinetic basis for discrimination by WT differs depending upon the DNA substrate. When G is 5’ to the templating base, WT discriminates correctly from incorrect dNTP by 11.8-fold at the level of Kt and 1.384-fold at the level of kpol as shown in the Table I. When T is 5’ to the templating base (Table II), discrimination against dCTP:T misinsertion by WT increases at the level of Kt and decreases at the level of kpol when compared with the DNA substrate in which G is 5’ to the templating base. A similar trend is observed for D246V.

This result prompted us to determine whether the misincorporation of dTTP opposite template G was altered by the base 5’ to the templating base. This time we changed the 5’G to A and measured the ability of D246V to misincorporate dTTP opposite G with this substrate. The data in Table II show that replacing the 5’G with an A results in 95.8-fold decrease in the
fidelity of misincorporation of dTTP opposite G. The kinetic basis for the decreased fidelity is lack of discrimination during ground state binding, $K_d$. As an internal control, we show that the fidelity of misinsertion of dGTP opposite G in this sequence context was similar for WT and D246V.

**D246V Does Not Extend Mispairs**—D246V is located on a solvent-exposed loop within the palm domain of pol. Another mutant that maps to this domain is E249K. This mutant has a propensity to extend mispaired primer termini (22). Therefore, we examined the ability of D246V to extend mispairs. As shown in Table III, the catalytic efficiency of D246V for extension of the mispaired primer termini dTTP:G and dTTP:C is similar to that of WT.

**D246V and WT Have Similar Binding Affinity for 1-bp Gapped DNA**—Active-site titrations were conducted as described under “Experimental Procedures” to measure the $K_{D_{DNA}}$ for formation of a productive complex between the gapped DNA substrate and D246V or WT. The $K_{D_{DNA}}$ values were 48.1 ± 11.0 and 13.6 ± 3.1 nM for WT and D246V, respectively. Thus, the dissociation constants for the two proteins showed only a 3.5-fold difference, suggesting that the Val substitution at amino acid residue 246 does not significantly impact upon the binding affinity of pol β to a gapped DNA substrate.

**DISCUSSION**

Kosa and co-workers (21, 26) isolated the D246V mutant as an AZT-resistant mutant by an *in vivo* selection method exploiting the idea that polymerase β can substitute for *E. coli* polymerase I. The steady-state kinetic data show that D246V is less efficient than WT for incorporation of AZT in a recessed primer template (21). In order to test the accuracy of the AZT-resistant pol β mutant enzyme, a missing base primer extension assay was carried out that shows that D246V is a mutator mutant opposite template C (22). Our data presented here show that when Asp-246 is altered to Val, the resulting enzyme is able to misincorporate nucleotides opposite all template bases with the exception of dATP opposite C. Misincorporation by D246V is affected by the base 5’ to the templating base, suggesting that the DNA assumes an altered position within the active site of D246V. Our data, combined with our previously published data on E249K (22), suggest that the
The best fit of the data to the hyperbolic equation. The data were fit to the single exponential equation to obtain $A$, opposite template C for WT.

From Tsai and co-workers (27) and shown in Scheme 1, is as follows. WT when the base 5' of dCTP opposite template T is 10-fold lower than that of (Table II). For example, the fidelity of D246V for misincorporation of dCTP opposite template T is described under "Experimental Procedures." The solid line represents the best fit of the data to the hyperbolic equation.

**D246V Cannot Discriminate during Ground State Binding—**The basic kinetic scheme of DNA polymerase β, as described by Tsai and co-workers (27) and shown in Scheme 1, is as follows. After initial complex formation with DNA, pol β binds to the dNTP substrate. This is reflected by the $K_D$. At this step, pol β selectively chooses a dNTP for insertion opposite a templating base. Ground state binding is followed by an isomerization of the polymerase ternary complex, which finally leads to a productive catalytic complex where chemistry occurs rapidly. This is described by $k_{pol}$. Each of these steps can play a major role for selection of the correct nucleotide. Our data shows that D246V has much less discrimination of nucleotides at the level of ground state binding when compared with WT.

**Misincorporation by D246V Is Affected by the 5'-Base to the Templating Base—**In this study we show that misincorporation of D246V is affected by the base 5' to the templating base (Table II). For example, the fidelity of D246V for misincorporation of dCTP opposite template T is 10-fold lower than that of WT when the base 5' to the templating base is G. When we altered the base 5' to the templating base to T (Table II), the fidelity of misincorporation of C opposite template T for D246V is only 3-fold less than WT. This suggests that when G is 5' to the templating base, it becomes the templating base, and dCTP is correctly incorporated opposite the G. The net result appears to be a misincorporation of dCTP opposite T. Further confirmation of our hypothesis is evidenced by the misincorporation of dTTP opposite G when the base 5' to the templating base is A. In this case there is a 100-fold decrease in the fidelity of D246V compared with WT. However, for dGTP misincorporation opposite G in the same sequence context, D246V and WT fidelities were similar. Therefore, our data suggest that the base 5' to the templating base becomes the actual templating base for the incoming nucleotide.

In each of the cases described above, D246V is unable to discriminate nucleotides during ground state binding. For example, in the case of misincorporation of dTTP opposite G, when A is 5' to the templating base, the $K_D$ for dTTP (11.5 μM) is lower than that for dCTP (59.9 μM). This suggests that D246V prefers to bind to dTTP versus dCTP. The same is true for the binding of dCTP opposite template T when G is 5' to the T. The simplest explanation for these results is that the base 5' to the templating base assumes the position of the templating base before or at the same time the nucleotide associates with the polymerase. We note that the equilibrium dissociation constant for DNA, $K_D$, for D246V and WT are similar. This suggests that altered DNA positioning within D246V does not result from more rapid dissociation and reassociation of D246V with the DNA, when compared with WT.

**The Flexible Loop Functions to Position the Primer—**The loop of the palm domain, consisting of amino acid residues 240–253 and connecting β-strands 4 and 5, is shown in Fig. 4. The loop itself is solvent-exposed in the crystal structure, and the lack of structural detail of this loop may suggest that it moves, even within the crystal. Our data suggest that alteration of the Asp-246 residue to the Val at the tip of the loop results in misincorporation resulting from an altered or misaligned DNA structure within the active site. The crystal structure shows that loop is positioned at a distance far from the active site, so close interaction of Asp-246 with the nucleotide or templating base in the active site is not likely. We suggest that this loop functions indirectly by maintaining the proper positioning or alignment of the DNA within the active site.

DNA polymerase β has two HhH motifs. One HhH motif is present within the 8-kDa domain of pol β, is composed of residues 1–78, 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 (17, 28). The second HhH motif acts as the primer grip. We suggest that the flexible loop may modulate the position of primer, resulting in primer mislocation as described by others in case of Y family polymerases (29, 30). As a result of primer mislocation, the base 5' to the templating base becomes the templating base.

**Perusal of the 1HUO and 1BPY structures (Protein Data base) of pol β show that the loop domain is disordered. Therefore, it is difficult at this time to interpret our data in terms of the structure of pol β. However, in the 1HUO structure, His-252 is ordered and appears to buttress against helix I, which contains residues 134–147. Helix I appears to push against the HhH in the thumb domain. This HhH interacts with the DNA primer. Alteration of Asp-246 to Val may alter the chemical nature of the loop, destabilizing its ability to buttress helix I and resulting in mislocation of the primer within the active site of D246V. It is also plausible that Asp-246 forms an electrostatic interaction with Lys-141 or Arg-137, which could result in stabilization of helix I and ultimately in maintaining the
primer grip in the correct position. Removal of the negative charge from Asp-246 would disrupt the interaction between residue 246 and residues on helix I. This could result in less stability of the primer grip and mislocation of the primer.

Sawaya et al. (13) have pointed out that upon binding of pol β to a single nucleotide gap, the DNA becomes kinked by about 90°. This results in stabilization of the templating base by stacking interactions with Lys-280 and stabilization of the exposed base pair downstream to the templating base by stacking interactions with His-34. These stacking interactions would most likely be disrupted in the D246V active site if the primer mislocates, especially in the case where the base 5’ to the templating base is able to base pair with the incoming dNTP. Once the primer mislocalizes it is possible that new stacking interactions occur between the new downstream base pair and the new templating base.

### Table I

| Sequence context of template | $k_{pol}$ | $K_d$ | $k_{pol}/K_d$ | $K_{di}/K_{di}'$ | $k_{pol}K_d$ | $F$ ($\times 10^3$/d) |
|------------------------------|-----------|-------|---------------|------------------|-------------|----------------------|
| $5'$-AC AA                   |           |       |               |                  |             |                      |
| 3'-TGGTTT                    | B-WT      | 24.6  | 1.1           | 11.1 ± 1.4       | 22.1 × 10^5 | 22.0                 |
|                              | T:A       | 0.064 | 0.006         | 635.0 ± 97.0     | 384.3       | 100.7                |
|                              | T:C       | 11.9  | 0.9           | 1911.1           | 126.0       | 121.7                |
| D246V                        | T:A       | 34.4  | 2.2           | 263.6 ± 6.2      | 2271.4      | 18.4 × 10^5          |
|                              | T:C       | 0.08  | 0.006         | 321.0 ± 56.7     | 430.0       | 17.2                 |
| 5'-AC TA                     | B-WT      | 6.7   | 0.3           | 2.0 ± 0.3        | 33.5 × 10^5 | 7.4                  |
| 3'-TGGAT                     | G-C       | 0.007 | 0.0015        | 245 ± 50         | 2233.3      | 6.1                  |
|                              | G:T       | 0.014 | 0.0001        | 111.7 ± 1.8      | 832.0       | 36.0                 |
|                              | G:G       | 0.008 | 0.0005        | 1081.5 ± 105.3   | 8850.0      | 1217.3               |

### Table II

| Sequence context of primer-template | $k_{pol}$ | $K_d$ | $k_{pol}/K_d$ | $K_{di}/K_{di}'$ | $k_{pol}K_d$ | $F$ ($\times 10^3$/d) |
|-------------------------------------|-----------|-------|---------------|------------------|-------------|----------------------|
| $\beta$-WT                           |           |       |               |                  |             |                      |
| C:G                                  | 11.7 ± 1.21| 89.3 ± 21| 2.4           | 28.5             |             | 1.3 × 10^5          |
| C:G                                  | 0.006 ± 0.040| 209.9 ± 50.9| 1950.0         | 2.4              |             | 1.3 × 10^5          |
| C:G                                  | 0.015 ± 0.0003| 100.0 ± 4.8| 780.0          | 1.1              |             | 150.0               |
| C:G                                  | 0.008 ± 0.0005| 161.1 ± 30.4| 1462.5         | 1.8              |             | 49.6                |
| C:G                                  | 34.4 ± 1.8 | 15.04 ± 2.1| 23 × 10^5      | 25.4             |             | 1.0                 |
| D246V                                |           |       |               |                  |             |                      |
| C:G                                  | 11.7 ± 1.21| 89.3 ± 21| 2.4           | 28.5             |             | 1.3 × 10^5          |

| Units are micromolar. |
| The $k_{pol}$ for correct (c) divided by incorrect (i). |
| The $K_d$ for incorrect (i) dNTP divided by correct (c). |
| Fidelity ($F$) was calculated as described under “Experimental procedures.” The substrates used in this experiment are 45TTT-U22-D22 and 45TTGGA-U22-D22 as shown in Fig. 1. |
| The templating base is underlined. |

| Sequence context of primer-template | $k_{pol}$ | $K_d$ | $k_{pol}/K_d$ | $K_{di}/K_{di}'$ | $k_{pol}K_d$ | $F$ ($\times 10^3$/d) |
|-------------------------------------|-----------|-------|---------------|------------------|-------------|----------------------|
| $\beta$-WT                           |           |       |               |                  |             |                      |
| C:G                                  | 11.7 ± 1.21| 89.3 ± 21| 2.4           | 28.5             |             | 1.3 × 10^5          |
| C:G                                  | 0.006 ± 0.040| 209.9 ± 50.9| 1950.0         | 2.4              |             | 1.3 × 10^5          |
| C:G                                  | 0.015 ± 0.0003| 100.0 ± 4.8| 780.0          | 1.1              |             | 150.0               |
| C:G                                  | 0.008 ± 0.0005| 161.1 ± 30.4| 1462.5         | 1.8              |             | 49.6                |
| C:G                                  | 34.4 ± 1.8 | 15.04 ± 2.1| 23 × 10^5      | 25.4             |             | 1.0                 |
| D246V                                |           |       |               |                  |             |                      |
| C:G                                  | 11.7 ± 1.21| 89.3 ± 21| 2.4           | 28.5             |             | 1.3 × 10^5          |

| Units are micromolar. |
| The $k_{pol}$ for correct (c) divided by incorrect (i). |
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| The templating base is underlined. |
fold over WT

D246V Mutant of pol β

To measure extension of a mispair, the rate of incorporation of the next correct nucleotide following the mispair was measured.

TABLE III

| Enzyme   | Terminal base pair   | $k_{pol}$ $M^{-1}s^{-1}$ | $K_d$ $μM$ | $k_{pol}/K_d$ $M^{-1}s^{-1}$ | $F$ ($×10^3$) | Fold over WT |
|----------|----------------------|--------------------------|------------|-------------------------------|----------------|--------------|
| β-WT     | G: C                 | 2.3 ± 0.07               | 134.2 ± 6.3| 0.17 $× 10^6$               | 0.06           | 1            |
| D246V    | G: C                 | 0.04 ± 0.001             | 136.5 ± 35.5| 293.0                        |                | 0.053        |
|          | G: T                 | 27.1 ± 3.6               | 661.9 ± 91.8| 0.48 $× 10^5$               | 2.4 $× 10^3$  | 1.5          |
|          | T: C                 | 0.028 ± 0.0007           | 30.7 ± 7.4 | 912.0                        |                | 1            |
| β-WT     | C: G                 | 4.2 ± 0.675             | 271.7 ± 53.6| 0.15 $× 10^5$               | 0.058          | 1.1          |
| D246V    | C: G                 | 0.007 ± 0.0008           | 722.5 ± 163| 9.6                          |                | 1.6          |
|          | C: T                 | 6.9 ± 0.52              | 57.2 ± 10.9 | $1.2 × 10^5$                | 50.9           | 2.4          |

* To measure extension of a mispair, the rate of incorporation of the next correct nucleotide following the mispair was measured.

* Fold over β-WT is calculated as Fidelity$_{D246V}$ / Fidelity$_{WT}$

The mutations in the loop domain are located at some distance from the active site of pol β which suggests that they exert their effect on enzyme-substrate interactions through an indirect mechanism. This is similar to known AZT resistance mutations in human immunodeficiency virus-reverse transcriptase, which produce long range changes in the active site rather than interacting directly with incoming nucleotides (33). Our previous studies (21) of steady-state kinetics of AZT resistance of D246V showed that it has less catalytic efficiency for AZT-TP incorporation opposite A. Our misincorporation data in the current study suggests that D246V misincorporates due to mislocation of the primer, which results in the base 5’ to the templating base becoming the templating base. Therefore, the less efficient incorporation of AZT-TP by D246V may be due to primer mislocation. The net result would be the formation of a nascent pair that is geometrically unfavorable, resulting in a low efficiency of AZT-TP incorporation. An alternative explanation is that altered positioning of the DNA within D246V in the presence of AZT-TP results in increased steric clash of AZT-TP with active site residues of pol β, resulting in inefficient incorporation. The present study reinforces the functional importance of residues distant from the active site of pol β by showing that a single amino acid alteration at the tip of flexible loop in the palm domain has a significant effect on polymerase fidelity.

Fig. 4. Crystal structure of pol β depicting the flexible loop. The protein backbone is in blue; the primer strand is in green; the template is red, and the ddCTP is in cream. The Asp-246, Glu-249, and His-252 residues of the flexible loop are in gray, as are Lys-141 and Arg-137 of helix I. The hairpin is part of the helix-hairpin-helix motif that forms the primer grip. See text for details on the proposed function of the flexible loop. The drawing was made using the coordinates of 1BPY in the Protein Data Bank with the INSIGHT II (version 2.0) program.

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