The E249K Mutator Mutant of DNA Polymerase β Extends Mispaired Termini*

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The DNA polymerase β mutant enzyme, which is altered from glutamic acid to lysine at position 249, exhibits a mutator phenotype in primer extension assays and in the herpes simplex virus-thymidine kinase (HSV-tk) forward mutation assay. The basis for this loss of accuracy was investigated by measurement of misincorporation fidelity in single turnover conditions. For the four misincorporation reactions investigated, the fidelity of the E249K mutant was not significantly different from wild type, implying that the mutator phenotype was not caused by a general inability to distinguish between correct and incorrect bases during the incorporation reaction. However, the discrimination between correct and incorrect substrates by the E249K enzyme occurred less during the conformational change and chemical steps and more during the initial binding step, compared with pol β wild type. This implies that the E249K mutation alters the kinetic mechanism of nucleotide discrimination without reducing misincorporation fidelity. In a missing base primer extension assay, we observed that the mutant enzyme produced mispairs and extended them. This indicates that the altered fidelity of E249K could be due to loss of discrimination against mispaired primer termini. This was supported by the finding that the E249K enzyme extended a G:A mispair 8-fold more efficiently than wild type and a C:T mispair 4-fold more efficiently. These results demonstrate that an enhanced ability to extend mispairs can produce a mutator phenotype and that the Glu-249 side chain of DNA polymerase β is critical for mispair extension fidelity.

DNA polymerase β has been implicated in base excision repair (3, 4) and appears to function during meiosis (5). Homozygous deletion of the pol β gene in mice is an embryonic lethal event (6), indicating that pol β is required during development, although its specific role is not clear. The best characterized function of pol β is gap-filling synthesis during base excision repair (BER). The BER pathway repairs oxidative and alkylate damage, as well as other DNA lesions. After excision of a damaged base by a DNA glycosylase, AP endonuclease (APE) binds to the abasic site and recruits pol β into a complex with the APE and DNA (7). APE incises the DNA, and pol β completes both the gap-filling synthesis and the deoxyribophosphatase steps (1, 8, 9). As AP sites are believed to occur in eukaryotic cells about 10,000 times per cell per day (10), this repair pathway is vital for genomic stability.

pol β may also have a role in DNA replication. It is required for conversion of single-stranded to double-stranded DNA in Xenopus extracts (11) and is able to join Okazaki fragments (12) and participates in the initiation of plasmid replication (13) in Escherichia coli. As pol β is not capable of processive synthesis of long stretches of DNA, it may contribute to replication in mammalian cells by specialized, small scale synthesis. pol β is the only known template-directed polymerase without homology to the replicative polymerases. The homology of pol β to the terminal transferase family of non-template-directed polymerases implies that its catalytic activity and template dependence evolved separately (14, 15). Since diverging from the ancestral terminal transferase-type gene, a relatively recent occurrence in evolutionary terms, pol β has acquired the ability to recognize and bind a template and to take orders from this new substrate in order to polymerize in a sequence-specific fashion. pol β therefore straddles two categories, the template-independent nucleotidyltransferases and the template-directed polymerases. As an enzyme with such an unusual evolutionary history, pol β is uniquely situated to shed light on the molecular basis of polymerase substrate specificity.

We previously described two AZT-resistant mutants of pol β identified by in vivo selection using the recA718 polA12 heterologous complementation system (12). In this system, pol β substitutes for DNA polymerase I in DNA replication at non-permissive temperature. The AZT-resistant mutants were selected from a randomly mutagenized library of pol β clones for their ability to grow on AZT in the recA718 polA12 strain at nonpermissive temperature (16). The first two AZT-resistant mutants we described, R253M and D246V, carried nonpermissive substitutions in the palm domain (16). The strong phenotypes exhibited in vivo and in vitro by the R253M and D246V mutants called attention to amino acid residues 240–253 that comprise a loop located within the palm domain of pol β, in which three different AZT resistance mutations were identified. Here we discuss the third of these palm domain loop substitutions, E249K, an active polymerase that exhibits a subtle AZT resistance in combination with a mutator phenotype.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The strain BL21 DE3 was used for protein expression and has genotype F′ ompT hsdSB (rB mB ) gal dcm (DE3).
The strain FT334, used to detect thymidine kinase mutations in the forward mutation assay, has genotype recA13 upp tdk.

Expression and Purification of Mutant Enzymes—WT and mutant proteins were expressed from the pHis-β vector, which expresses pol β as a fusion protein with a six-residue poly-histidine tag at the N terminus. The enzymes were purified as described previously, using Ni²⁺-charged His-bind resin from Novagen or Ni²⁺-nitrilotriacetic acid resin from Qiagen (16).

For large scale preparations, fast protein liquid chromatography-driven Ni²⁺ columns were used (Hi-Trap chelating resin, Amersham Pharmacia Biotech). In this case, the same procedure was followed except that the protein was eluted with an imidazole gradient rather than a single step.

HSV-th Forward Mutational Assay—The in vitro mutation frequencies were obtained using the method of Li et al. (17). The template plasmid has a 203-base gap in the HSV-thymidine kinase gene which is filled in by pol β. Mutation frequency was calculated as described by Eckert et al. (18).

A2T Incorporation Assay—The ability of each enzyme to incorporate dNTPs into a primer-template was tested in steady-state incorporation reactions using annealed 16- and 45-mer as described previously (16). Reactions were conducted at 37 °C in 50 mM Tris, pH 8.0, 10 mM MgCl₂, 20 mM NaCl, 2 mM DTT, 0.2 mg/ml BSA, 2.5% glycerol, and 200 nM 32P end-labeled primer-template. The reactions 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 0–5 s.

The kinetics of misincorporation were determined manually under the above single turnover conditions. Reactions were performed by preincubating 750 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 0–2 mM, and reaction times were 0–1200 s.

The data were fit by nonlinear regression using the program SigmaPlot version 4.14 (Jandel Scientific). The data from the single-turnover experiments were fit with a single exponential equation: [product] = A(1 – exp(–kₘᵦₜ))t, where A is the amplitude and kₘᵦₜ is the observed first order rate constant for dNTP incorporation. To obtain kₘᵦ, the equilibrium dissociation constant, and kₘᵦ, the maximum rate of polymerization, the data were fit with the hyperbolic equation: kₘᵦ = kₘᵦ(1 + [dNTP]/Kₛₕₕₜₜ)^[dNTP]]. Fidelity values were calculated using the following equation: fidelity = ((kₘᵦ/dNTP)/kₘᵦ)correct + ((kₘᵦ/dNTP)/kₘᵦ)incorrect/kₘᵦ/Kₛₕₕₜₜ.

Single Turnover Mispair Extension Assays—These assays were performed as described above, in single turnover conditions, except the primer-template contained mispaired termini.

RESULTS

A High Activity, Low Fidelity Mutant—The alteration of Glu-249 to Lys was initially identified by an in vivo selection for AZT-resistant mutants of pol β (16). This substitution produced a weak AZT resistance phenotype when expressed in SC18–12 bacteria. pol β E249K appeared to incorporate AZT-TP in vitro about as efficiently as β-WT (Fig. 2), which was unsurprising due to the weakness of the in vivo phenotype. In contrast, the previously identified R253M and D246V mutants exhibited profound in vivo phenotypes and significantly reduced AZT-TP incorporation in vitro (16).

In order to investigate the accuracy of the AZT-resistant pol β mutant enzymes, the ability of β-WT to misincorporate and extend incorrect bases in a missing base assay was compared with the E249K, R253M, and D246V mutants. In this assay three of the four dNTPs are present. Because copying the template requires that the enzyme misincorporate and extend when the correct base is not present, error-prone enzymes will extend the primer farther than enzymes with high fidelity. Fig. 3 shows that the E249K mutant extended the primer substantially farther than the β-WT enzyme, implying that it is more capable of inserting and extending incorrect bases. Whereas R253M and D246V did extend farther than β-WT in some reactions, these differences were small and not consistently reproducible, unlike E249K. E249K was more effective than β-WT in all PCR reaction, but the difference was most pronounced in the A- and C- reactions, representing misincorporation opposite T and G. pol β E249K, unlike β-WT, was able to extend the primer past multiple misinsertion sites, indicating that the mutant enzyme is more willing to extend mispaired termini. The increase in misincorporation and mispair extension suggested that the fidelity of the E249K mutant

![Table](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAQ8AAAAYCAYAAACWZKpRAAABlUlEQVQ4y337jMz8/p6M4CwAIA0LgQkAAgAAAAAElFTkSuQmCC)

![Graph](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAgkAAAYCAYAAACWZKpRAAABlUlEQVQ4y337jMz8/p6M4CwAIA0LgQkAAgAAAAAElFTkSuQmCC)
E249K Mutator Mutant

The E249K mutant enzyme catalyzes the synthesis of DNA with lower fidelity than β-wt.

| Experiment | Frequency × 10⁻⁴ | Fold over WT |
|------------|-----------------|-------------|
| β-WT       | 13              | 1           |
| E249K      | 360             | 28          |

WT is pol β wild type. The forward mutation assays were performed as described by Li et al. (17).

which selects for colonies with an inactive tk gene. As shown in Table I, the mutation frequency for E249K was between 19- and 28-fold increased over that of β-WT. This result indicates that the E249K mutation lowers the fidelity of pol β.

**Mispair Fidelity on a 3’-Recessed Template**—To investigate the fidelity of the E249K mutant on a 3’-recessed template, we measured the equilibrium dissociation constants, Kₜ, and the maximum rates of polymerization, kₚₒˡ, during incorporation of incorrect dNTPs and their corresponding correct dNTPs on the 45–15-mer and 45–14-mer oligonucleotides shown in Fig. 1. In the case of misincorporation reactions, where the time points are relatively long, n + 2 and n + 3 products were often visible, particularly at high substrate concentrations (Fig. 4). This does not interfere with measurement of the first incorporation, as all extension products can be quantified and summed together. However, the mutant did appear to generate qualitatively more of these secondary products. On the 45–15-mer template, after the C:T misincorporation the next two incorporations are T:T and A:T, and mostly the latter product (the n + 3 band) accumulates, presumably because the A:T incorporation is comparatively rapid. Because the enzyme is present in large excess over DNA (15:1 molar ratio), and the products appear only in later time points when most of the primer has been extended, the n + 2 and n + 3 products are most likely the result of multiple binding events to the same primer-template molecule.

Surprisingly, no significant difference between WT and mutant enzymes was apparent in the C:T misincorporation reaction. Time courses of product formation of dTTP insertion opposite template C with different concentrations of substrate are shown in Fig. 5A. The observed rates of product formation (kₒₛ) were determined as described under “Experimental Procedures” and were plotted against the various concentrations of dTTP used in these experiments, as shown in Fig. 5B. The Kₜ and kₒˡ values for misincorporation of T opposite C and A opposite G on a recessed primer-template were calculated as described under “Experimental Procedures” and are shown in Table II. The E249K enzyme discriminates as effectively as β-WT against incorporation of dTTP opposite C, having a fidelity of 1 × 10⁵, which is 4-fold greater than that of β-WT. For misincorporation opposite C, β-WT has a discrimination factor of 8500 (68/0.008) between the correct and incorrect substrates at the level of kₒˡ, and only a 2-fold (317/132) discrimination factor in Kₜ. This indicates that β-WT discriminates correct from incorrect dNTP substrate mostly at the level of kₒˡ. The E249K mutant discriminates correct from incorrect substrate by a factor of 6700 at the Kₜ level, which is similar to β-WT. However, the discrimination factor of E249K for correct versus incorrect at the initial binding step is 14, which is 7-fold greater than observed for β-WT.

The results of the G:A fidelity experiments, using the 45–14-mer 3’-recessed template (Table II), were similarly surprising. In this case, the E249K mutant did exhibit a 3.7-fold increase in the catalytic efficiency of misincorporation. However, the misincorporation fidelity of the E249K enzyme was not significantly different from β-WT, because the efficiency of correct G:C incorporation also increased slightly. The discrimination

enzyme is altered.

**Mutator Phenotype of pol β E249K**—To measure the fidelity of DNA synthesis by the possible mutator enzyme E249K, we employed the HSV-tk forward mutation assay developed by Eckert et al. (18). In this assay, pol β is required to fill a gap 203 bases long in the HSV-tk gene. The resulting pool of plasmid molecules is transformed into E. coli which lack endogenous thymidine kinase activity. Transformants are plated in the presence and absence of the drug 5’-fluoro-2’-deoxyuridine, ()

![FIG. 2. Incorporation of AZT by β-WT and E249K.](image)

![FIG. 3. The E249K mutant enzyme is able to misincorporate and extend mismatches in a missing base assay.](image)
WT

The E249K enzyme produces n + 2 and n + 3 products in a primer extension assay. The 45–15-mer primer-template was employed in the experiment shown. Reactions were conducted at 37 °C in 50 mM Tris, pH 8.0, 10 mM MgCl₂, 20 mM NaCl, 2 mM DTT, 0.2 mg/ml BSA, 2.5% glycerol, 50 mM β-mercaptoethanol, and 750 nM enzyme. 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 0–5 s. The kinetics of misincorporation were determined manually under the above single turnover conditions. Reactions were performed by preincubating 750 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 0–2 mM, and reaction times were 0–1200 s. The base pairs formed after misincorporation are on the right.

Factors at the level of kₙₚₐₙ for β-WT and E249K are 1500 and 500, respectively, and for Kₐ are 10 and 22, respectively. The E249K mutant discriminates against misincorporation at the level of Kₐ but more at the level of Kₙₚₐₙ. This results in E249K having a similar fidelity to that of β-WT but suggests that discrimination of correct from incorrect dNTP occurs by a different kinetic mechanism than that of β-wt.

Kinetics of Misincorporation on Single Base Gap Templates—Next, we compared the fidelity of E249K to that of β-WT on a single base gap with a 5′-phosphate group because this DNA substrate is the preferred template of pol β (2, 21). The kinetic constants obtained from analyzing the misincorporation of T opposite C and G opposite T on a 1-base pair gapped DNA substrate are shown in Table II. The kₚₐₙ and Kₐ for T:G misincorporation were similar for the mutant enzyme compared with β-WT, as were the catalytic efficiencies and fidelities.

Discrimination against the incorporation of dTTP opposite C by the β-WT enzyme seems to occur predominantly at the kₚₐₙ level, where the correct nucleotide is favored by 2700-fold, as shown in Table II. Ground-state nucleotide binding contributes only 19-fold discrimination. The E249K mutant discriminates against misincorporation of dTTP opposite C almost as effectively as β-wt, but does so less than β-WT at the kₚₐₙ stage (390-fold) and more at the binding stage (70-fold). This implies that the E249K mutation alters the kinetic mechanism of nucleotide discrimination without reducing misincorporation fidelity.

Kinetics of Extension of a Mismatched 3′ Terminus—The apparent willingness of the E249K enzyme to perform successive misincorporations in both the missing base and the primer extension assays led us to examine the mispair extension fidelity of β-WT and E249K. We measured the ability of β-WT and E249K to incorporate a correct nucleotide (C:G) following a G:A or T:C mispair. The mispair extension reaction was slow for both enzymes, as shown in Table III. The E249K mutant did extend both mismatches more efficiently than β-WT. The difference was more pronounced with the purine:pyrimidine mismatch, for which the E249K mutant catalytic efficiency was increased by more than 8-fold, compared with 4-fold for the pyrimidine:pyrimidine mispair. Surprisingly, the increased efficiency of the E249K enzyme appeared to be governed by different kinetic parameters for the two mispairs. The mutant enzyme’s kₚₐₙ for incorporation of C:G following a G:A mismatch was increased 1.8-fold, and Kₐ(dGTP) was decreased 4.5-fold, compared with β-WT. The difference in C:T mispair extension was due to a higher kₚₐₙ (4-fold) of the mutant enzyme.

### DISCUSSION

The E249K Mutator Mutant Exhibits High Misincorporation Fidelity and Possibly Increased Affinity for Nucleotide Substrate—The E249K mutant enzyme demonstrated a loss of accuracy in the missing base primer extension assay and in the HSV-tk forward mutation assay. This phenotype often indicates an inability to discriminate against incorrect nucleotides during incorporation. However, using two 3′-recessed and two gapped primer-templates, the E249K mutant failed to demonstrate a reduced misincorporation fidelity to account for its apparent mutator phenotype. These results lead to the conclusion that the mutator phenotype of this mutant is not caused by a global loss of misincorporation fidelity. Without testing all possible mispairs, it is impossible to say whether the mutant enzyme has lower fidelity for some mispairs, but the lack of compromised fidelity for C:T, T:G, and G:A mispairs is difficult to reconcile with the evidence that this mutant is in fact error-prone.

During all four correct incorporation reactions, the mutant was at least as efficient as β-WT, as measured by kₚₐₙ/Kₐ. The Arg-283 → Ala mutant exhibits dramatically reduced fi-
EF249K Mutator Mutant

Phenotype of pol β EF249K—The possibility that the EF249K enzyme was mispair extension-proficient was suggested by the consistent appearance of \(n + 2\) and \(n + 3\) bands in misincorporation reactions (Fig. 4). The formation of \(n + 3\) product requires the enzyme to perform two successive misincorporations followed by one correct incorporation. In our analysis of the mispair extension efficiencies of β-WT and EF249K, we found that EF249K was more proficient than β-WT in extending mispaired termini. This indicates that the Glu-249 amino acid residue is critical for the prevention of mispair extension.

The EF249K mutant, like the β-WT enzyme, is far more capable of extending a correctly paired terminus than a mispaired one. For both enzymes, the efficiency of a normal extension is several thousand-fold higher than a mispair extension. However, the ratio of catalytic efficiencies, sometimes known as mispair extension fidelity, is not analogous to misincorporation fidelity. When a polymerase is bound to template in the presence of correct and incorrect dNTPs, the substrates can compete for incorporation. Therefore, the ratio of catalytic efficiencies for correct and incorrect incorporations can be expected to reflect the relative frequency with which the polymerase will “choose” correct and incorrect nucleotides. That allows misincorporation fidelity, defined as ratio of catalytic efficiencies, to be a reliable indicator of error rates. This reasoning does not apply to mispair extension. Once an incorrect nucleotide has been incorporated in a cell or in a typical in vitro polymerase reaction, the mismatched terminus is not in competition with identical but correctly matched termini. If the polymerase were presented with such equivalent templates, it could be expected to “choose” to extend the correct terminus more frequently, but the ratio would be controlled by the DNA binding affinity of the enzyme (24) which is eliminated from single turnover reactions by pre-binding excess enzyme with DNA as well as by the mispair extension fidelity. This alone could complicate the interpretation of mispair extension fidelity measurements, but in addition, such direct competition between correct and incorrect termini is probably not representative of relevant polymerase reactions. Instead, in the forward mutation assay, the polymerase faced with a mismatched terminus can only choose to extend it or leave it as it is. The efficiencies of mispair extension suggest that the EF249K mutant will choose to extend far more frequently than β-WT. Therefore the large increase in the efficiency of mispair exten-

### Table II

| Enzyme | Template | Base pair | \(k_{\text{pol}}\) | \(K_d\) | \(k_{\text{pol}}/K_d\) | Fidelity |
|--------|----------|----------|---------------|--------|-----------------|----------|
| β-WT   | Recessed | C:G      | 68 ± 11       | 122 ± 41 | 5.2 × 10^3      | 2.4 × 10^4 |
| E249K  | Recessed | C:T      | 0.008 ± 0.0007 | 217 ± 92 | 21.3            | 2.4 × 10^4 |
| β-WT   | Recessed | C:G      | 88 ± 12       | 40 ± 9  | 2.2 × 10^5      | 1.0 × 10^5 |
| E249K  | Recessed | C:T      | 0.013 ± 0.002 | 591 ± 175 | 21.4            | 2.4 × 10^4 |
| β-WT   | Recessed | G:G      | 8.9 ± 0.5     | 63 ± 20  | 1.4 × 10^8      | 1.5 × 10^4 |
| E249K  | Recessed | G:G      | 0.006 ± 0.001 | 616 ± 50 | 9.4             | 4.5 × 10^4 |
| β-WT   | Gapped   | T:A      | 48 ± 2.0      | 27 ± 4   | 1.8 × 10^10     | 2.0 × 10^4 |
| E249K  | Gapped   | T:A      | 64 ± 4.4      | 52 ± 7.5 | 4.9 × 10^10     | 4.9 × 10^4 |
| β-WT   | Gapped   | T:G      | 17 ± 0.13     | 588 ± 78 | 2.8 × 10^13     | 1624     |
| E249K  | Gapped   | C:T      | 81 ± 10       | 81 ± 23  | 1.0 × 10^10     | 1.0 × 10^4 |
| β-WT   | Gapped   | C:T      | 0.03 ± 0.0007 | 1551 ± 842 | 32.2           | 5.2 × 10^4 |
| E249K  | Gapped   | C:T      | 39 ± 4.5      | 25 ± 7   | 1.4 × 10^6      | 51.6     |

### Table III

| Enzyme | Terminal mispair | \(k_{\text{pol}}\) | \(K_d\) | \(k_{\text{pol}}/K_d\) |
|--------|-----------------|---------------|--------|-----------------|
| β-WT   | G:A             | 0.06 ± 0.008  | 780 ± 22 | 80               |
| E249K  | G:A             | 0.110 ± 0.008 | 174 ± 45 | 640              |
| β-WT   | T:C             | 0.03 ± 0.002  | 260 ± 30 | 110              |
| E249K  | T:C             | 0.12 ± 0.02   | 260 ± 12 | 420              |

To measure extension of a mispair, the rate of incorporation of the next correct nucleotide following the mispair was measured. To measure extension of the G:A mispair the 45–15A oligonucleotide was employed, and for the T:C mispair the 45–15C oligonucleotide was used. The next correct base pair after G:A was G:G and after C:T was C:G.

Fold over β-WT is calculated as \((k_{\text{pol}}/K_d)_{\text{E249K}}/((k_{\text{pol}}/K_d)_{\text{β-WT}})\). All experiments were conducted at least three times.

* S.-X. Li, A. M. Shah, K. S. Anderson, and J. B. Sweasy, submitted for publication.

### Table IV

| Enzyme | Terminal mispair | \(k_{\text{pol}}\) | \(K_d\) | \(k_{\text{pol}}/K_d\) |
|--------|-----------------|---------------|--------|-----------------|
| β-WT   | G:A             | 0.06 ± 0.008  | 780 ± 22 | 80               |
| E249K  | G:A             | 0.110 ± 0.008 | 174 ± 45 | 640              |
| β-WT   | T:C             | 0.03 ± 0.002  | 260 ± 30 | 110              |
| E249K  | T:C             | 0.12 ± 0.02   | 260 ± 12 | 420              |
sion by the mutant enzyme is highly significant and may underlie the mutator phenotype of E249K.

Wild-type pol β, like other polymerases, is known to be inefficient at extending mismatched termini (22), but the effect of various mispairs on mispair extension fidelity has not been characterized. A tendency to extend mispaired bases could elevate the mutation frequency in the forward mutation assay by sealing misincorporation errors into the nascent strand. Because pol β is a distributive polymerase, it is unlikely that the polymerase completely fills the 203-nucleotide gap in the HSV-1k forward mutation assay during one DNA binding event. It is more likely that pol β binds to the DNA, catalyzes the addition of up to 9 bases, and dissociates (25); the rest of the gap is most likely filled by other polymerase molecules present in the reaction. If an error is committed during one round of DNA synthesis, the only way in which that particular gap can be filled is by the extension of a mispair. The E249K mutant enzyme, by extending a greater proportion of the mismatched termini compared with β-WT, might then seal in errors, resulting in more mutations that are detectable. This could explain the dramatic increase in mutation frequency seen in the HSV-1k forward mutation assay.

A mismatch extension phenotype for E249K would also help to explain the results of the missing base primer extension assay (Fig. 2), where synthesis of longer products requires the insertion of incorrect bases followed by extension of the mispairs. A precedent for infidelity by mispair extension exists in wild-type HIV-reverse transcriptase, which produces error-prone replication largely by its enthusiasm for extending mispaired termini (26).

A reluctance to extend mispairs is an important part of the fidelity of DNA polymerase I family and DNA polymerase α family polymerases (24, 27, 28). By slowing the extension of a mismatch, these polymerases allow time for their exonuclease activities to remove the error (29). This kinetic coupling of inefficient mispair extension to exonuclease degradative activity is an important fidelity mechanism and requires a delicate balance between polymerase and nuclease rates. Because pol β does not possess exonuclease activity, this coupling is not an available mechanism for pol β fidelity. This could produce evolutionary pressure on pol β to reject mispaired termini, in order to avoid sealing in errors, or to extend them more readily, in order to avoid the persistence of unrepaired AP sites.

For β-WT, the presence of a mismatched terminus may slow extension due to an inability of the enzyme to bind to the mismatched DNA. Alternatively, the rate-limiting step during mispair extension by β-WT may occur during active site chemistry or the presence of a mismatch may promote rapid dissociation of the ternary complex. Thus, extension of a mismatched terminus by the E249K enzyme may result from a higher binding affinity of the E249K enzyme for mismatched DNA or from an alteration in active site chemistry or the accommodation of a mismatch within the active site such that the ternary complex is more stable than it is with β-WT. A more detailed study of the reaction mechanism for β-WT and E249K should pinpoint the rate-limiting step of the reaction with a mispair in the active site.

The Role of a Flexible Loop in the Palm Domain—Selection for AZT resistance identified three mutations within the 240–253 loop of the palm domain of pol β (16). The finding that three different residues on this loop can participate in AZT resistance or fidelity suggests that the loop is important for polymerase function and particularly for substrate specificity. The increased affinity for nucleotide substrates exhibited by the E249K mutant may implicate this loop in ground-state nucleotide binding. The 240–253 loop connects β-strands 4 and 5 as shown in Fig. 6; strand 5 also includes the catalytic residue Asp-256 (not shown). The loop itself is disordered or solvent-exposed in crystal structures and is positioned at some distance from the active site (30, 31). This makes it difficult to deduce what the role of this loop in substrate interactions could be. However, the loop is probably flexible, given the lack of secondary structure apparent in the crystal structures, and its position in crystals is therefore not necessarily physiologically relevant.

The pol β protein bound to DNA resembles a donut shape, with the DNA entering and leaving the active site (donut hole) from one face. The nucleotide is believed to enter the cleft from the opposite face, due to the obstruction of the first face by the DNA (32). The 240–253 loop protrudes from the donut on the substrate-entrance face, putting it in a plausible position to influence the approach of substrate to the channel. This influence could take place directly, if the loop managed to fold up to form a channel gate, although it does not appear long enough to do so, barring other conformational changes. A gating effect could also occur less directly, through alteration of intramolecular contacts that propagate distant changes to the active site.

We have previously speculated that the loop is the site of protein-protein interactions (16). An example of such a scenario is as follows: suppose that the loop were the site of an inhibitory interaction with XRCC1 in vivo. The binding of XRCC1 “pulls” on the loop, distorting the conformation of the active site, or the channel opening, in such a way as to discourage nucleotide binding. The enzyme is now switched off and therefore prevented from conducting strand displacement synthesis. An AZT resistance mutation such as R253M causes the switch to assume a partially off conformation, discouraging the binding of AZT-TP. The E249K mutation, however, turns the switch on, perhaps to an even more favorable position than the normal wild-type configuration, allowing the more efficient binding of nucleotide. As yet, no experimental evidence exists for this proposition, but a distortion of this nature would explain why such a mutation could affect pol β function even in E. coli, where the normal protein regulators are probably absent, and in vitro.

The disordered structure of residues 240–253 in the β-WT
crystal makes it difficult to propose a role for this loop in the prevention of mispair extension. The role of the loop in the prevention of mispair extension may be important to maintain optimum active site geometry because the loop is located within the palm domain and could form part of the floor of the active site of pol β. Alteration of a residue of this loop may disrupt the packing of the floor of the active site which could alter the geometry of the active site, allowing it to accommodate a mispair. However, the role of the loop in the prevention of mispair extension must await further study.

**Substrate Choice in Fidelity and Drug Resistance**—The mutator mutations, like the other AZT resistance mutations identified by the selection, are located at some distance from the active site of pol β. This requires that they exert their effect on enzyme-substrate interactions through an indirect mechanism. In this they resemble some of the known AZT resistance mutations in HIV-reverse transcriptase, which produce long range changes in the active site rather than interacting directly with incoming nucleotides (33–35). Crystal structures of the quadruple mutant Asp-67 → Asn, Lys-70 → Arg, Thr-215 → Phe, and Lys-219 → Gln have demonstrated that distant changes can produce drug resistance through changes in template interactions or through subtle changes in conformation that are propagated to the active site. Therefore, remote control of substrate choice by amino acids distant from the binding pocket may be a general property of DNA polymerases.

In that sense, the selection successfully imitates nature. Just as a virus requires reverse transcriptase activity for survival, the heterologous complementation system insists on active pol β for bacterial growth. The appearance of mutations in residues that do not directly interact with substrate is probably not an anomaly but an indication that those residues that do interact directly with DNA and substrate are critical and cannot be mutated without crippling the enzyme. 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.

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**REFERENCES**

1. Singhal, R. K., Prasad, R., and Wilson, S. H. (1995) J. Biol. Chem. 270, 949–957
2. Chagovetz, A. M., Sweasy, J. B., and Preston, B. D. (1997) J. Biol. Chem. 272, 27561–27564
3. Clairmont, C. A., and Sweasy, J. B. (1996) J. Bacteriol. 178, 656–661
4. Sobol, R. W., Horton, J. K., Kuhn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K., and Wilson, S. H. (1996) Nature 379, 183–186
5. 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
6. Gu, H., Mart, J., Orban, P., Possmann, H., and Rajewsky, K. (1994) Science 265, 102–106
7. Bennett, R. A. O., Wilson, D. M., III, Wong, D., and Demple, B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7166–7169
8. Srivastava, D. K., Vande Berg, B. J., Prasad, R., Molina, J. T., Beard, W. A., Tomkinson, A. E., and Wilson, S. H. (1998) J. Biol. Chem. 273, 21293–21299
9. Matsumoto, Y., and Kim, K. (1995) Science 269, 699–702
10. Dianov, G., and Lindahl, T. (1994) Curr. Biol. 4, 1069–1076
11. Jenkins, T. M., Saxena, J. K., Kumar, A., Wilson, S. H., and Ackerman, E. J. (1992) Science 258, 475–478
12. Sweasy, J. B., and Loeb, L. A. (1992) J. Biol. Chem. 267, 1407–1410
13. Sweasy, J. B., Chen, M., and Loeb, L. A. (1995) J. Bacteriol. 177, 2923–2925
14. Matsukage, A., Nishikawa, K., Ohi, T., Seta, Y., and Yamaguchi, M. (1987) J. Biol. Chem. 262, 8960–8962
15. Anderson, R. S., Lawrence, C. B., Wilson, S. H., and Beattie, K. L. (1987) Gene (Amst.) 60, 163–173
16. Kosa, J. L., and Sweasy, J. B. (1999) J. Biol. Chem. 274, 3581–3588
17. Li, S.-X., Vaccaro, J. A., and Sweasy, J. B. (1999) Biochemistry 38, 4800–4808
18. Eckert, K. A., Hile, S. E., and Vargo, P. L. (1997) Nucleic Acids Res. 25, 1450–1457
19. Suzuki, M., Avicola, A. K., Hood, L., and Loeb, L. A. (1997) J. Biol. Chem. 272, 11228–11235
20. Johnson, K. A. (1995) Methods Enzymol. 249, 38–61
21. Singhal, R. K., and Wilson, S. H. (1995) J. Biol. Chem. 269, 15906–15911
22. Ahn, J., Werneburg, B. G., and Tsai, D. M. (1997) Biochemistry 36, 1100–1107
23. Carson, M. (1991) J. Appl. Crystallogr. 24, 958–961
24. Ehols, H., and Goodman, M. F. (1991) Annu. Rev. Biochem. 60, 477–511
25. Oprea, P. L., Sweasy, J. B., and Eckert, K. A. (1998) Biochemistry 37, 2111–2119
26. Perrino, F. W., Preston, B. D., Sandell, L. L., and Loeb, L. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3843–3847
27. Perrino, F. W., and Loeb, L. A. (1989) J. Biol. Chem. 264, 2988–2995
28. Mendeloff, L. V., Petruska, J., and Goodman, M. F. (1990) J. Biol. Chem. 265, 2338–2346
29. Kuchta, R. D., Benkovski, P., and Benkovic, S. J. (1988) Biochemistry 27, 6716–6725
30. Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) Biochemistry 36, 11205–11215
31. Pelletier, H., Sawaya, M. R., Wolfe, W., Wilson, S. H., and Kraut, J. (1996) Biochemistry 35, 12742–12761
32. Beard, W., and Wilson, S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7863–7867
33. Stein, C. A., Levin, D., and Oxford, J. S. (1994) J. Med. Virol. 44, 115–121
34. Kohlstad, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) Science 256, 1783–1790
35. Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Beyer, P. L., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A., and Arnold, E. (1994) J. Mol. Biol. 243, 369–387