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†

Patricia L. Opresko,‡ Joann B. Sweasy,§ and Kristin A. Eckert*,‡

Department of Biochemistry and Molecular Biology and The Jake Gittlen Cancer Research Institute, Pennsylvania State University College of Medicine, M. S. Hershey Medical Center, P.O. Box 850, Hershey, Pennsylvania 17033, and Departments of Therapeutic Radiology and Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520

Received September 11, 1997; Revised Manuscript Received December 4, 1997

ABSTRACT: This study describes the first complete in vitro error specificity analysis of a mutator DNA polymerase that is altered in a residue not predicted to contact either the DNA or dNTP substrate. We examined this mutator form of polymerase β (Y265C) in order to elucidate the critical role tyrosine 265 plays in the accuracy of DNA synthesis. Our results demonstrate that an increase in both frame shift errors in homonucleotide repeat sequences and base substitution errors contribute nearly equally to the Y265C mutator phenotype. The models described for production of these errors, primer/template misalignment and base misincorporation, respectively, are distinctly different, suggesting the Y265C alteration affects discrimination against both types of error production pathways. In addition, Y265C displays a 530-fold increase in multiple errors within the 203-base pair target region examined, relative to that of wild type. Processivity studies revealed that Y265C retains the near distributive nature of DNA synthesis characteristic of the wild type polymerase β. Therefore, multiple errors exhibited by Y265C most likely result from independent polymerase binding events. Localization of tyrosine 265 in the X-ray crystallographic structure suggests this residue may play a role in mediating a conformational change of the polymerase [Pelletier, H., et al. (1996) *Biochemistry 35*, 12742−12761]. A conformational change is predicted to enhance the accuracy of DNA synthesis by imposing an induced fit selection against premutational intermediates. The observed loss of discrimination against both misalignment-mediated and misincorporation-mediated errors produced by polymerase Y265C is consistent with such a model.

The accuracy of the DNA polymerases involved in DNA repair and replication is important for maintaining the integrity of the genome in both eukaryotes and prokaryotes. Detailed kinetic studies of DNA synthesis by several polymerases describe various biochemical stages of DNA polymerase error discrimination, and indicate that polymerases differ in the relative contribution of each step to the overall fidelity of the reaction (*1*−*6*). The first stage of error discrimination occurs at the nucleotide binding level (ground state selectivity) and is a function of the free energy of base pairing between the incoming nucleotide and the template base. A second stage, induced fit selectivity, is imposed by a conformational change in the polymerase prior to chemistry which may be inhibited by the presence of mispairs (*4*). Differential rates of phosphodiester bond formation for correct and incorrect base pairs is an additional mechanism of error discrimination (*1*). Although kinetic analyses have provided biochemical information about the mechanisms DNA polymerases utilize for accurate DNA synthesis, structural information concerning the specific amino acid residues involved in these processes is limited.

X-ray crystal structures elucidated for Klenow fragment, *Thermus aquaticus* DNA polymerase, HIV-1 reverse transcriptase (RT), polymerase β, and bacteriophage RB69 DNA polymerase (*7*−*11*), reveal that DNA polymerases share common structural features. Therefore, structure−function studies based on more simple polymerases are likely to provide information relevant to all polymerases (*12*). These structures have allowed identification of residues predicted to contact both the DNA and dNTP substrates, and therefore have been important for structure−function studies designed to elucidate the role these residues may play in the fidelity of DNA synthesis. Alteration of residues in the subdomain predicted to contact the primer/template DNA results in an increase in frame shift errors presumably by primer/template misalignment. Examples of these polymerase mutators include G262A and Y266A mutants of HIV-1 RT (*13*) and a derivative of Klenow polymerase that is missing residues in helix H1 (*14*). In contrast, alterations in residues predicted to contact or position the incoming dNTP result in a decrease in base substitution fidelity due to an increase in nucleotide misincorporation. Examples of these mutators include

† This research was supported by Research Project Grants CN144 (to K.A.E.), NP930 (to J.B.S.), and JFRA (to J.B.S.) from the American Cancer Society.

* To whom correspondence should be addressed. Telephone: 717-531-4065. Fax: 717-531-5634.

‡ Pennsylvania State University College of Medicine.

§ Yale University School of Medicine.

S0006-2960(97)02271-X CCC: $15.00
Published on Web 02/04/1998

Y766A and Y766S of Klenow polymerase (*15*, *16*), and Y865S of human polymerase α (*17*). In addition, alterations in HIV-1 RT residues predicted to interact with either the incoming dNTP (Y115) or the sugar moiety of either the primer nucleotide or the dNTP (M184) result in increased misinsertion errors (*18*, *19*). Polymerase β mutants have been constructed utilizing the X-ray crystal structure prediction that residues N279, Y271, and R283 are within hydrogen bonding distance of the incoming dNTP, the terminal primer nucleotide and the templating nucleotide, respectively (*10*). Mutants which alter the R283 residue (A > L > K) result in significantly reduced catalytic activity and misinsertion fidelity, while frame shift fidelity has yet to be examined (*6*, *20*).

We have used an alternative approach based on a genetic screen in *Escherichia coli* to identify residues important for accurate polymerase β DNA synthesis (*21*). We have found a class of mutator polymerases with amino acid changes in structural regions not predicted to directly contact substrate (*21*). Two of the isolated mutators have a single amino acid change at position 265 located in a hinge region connecting two subdomains, which is predicted to mediate a conformational change (*10*). One of these mutant polymerases, Y265C,[1] confers a 30-fold increase in mutation frequency measured in *E. coli*, relative to that of wild type polymerase β (β-wt) (*21*). Kinetic studies demonstrated that this mutator is catalytically active, although the steady state catalytic rate (k_{cat}) and $K_{M(DNAactivated)}$ are 20- and 5-fold lower, respectively, compared to those of β-wt (*21*). In addition, in vitro reversion mutagenesis assays suggested that the frequency of frame shift and base substitution errors at a single site was increased for Y265C, relative to that of β-wt (*21*). In this study, we used an in vitro forward mutagenesis assay to determine the specific types of frame shift, base substitution, and other errors Y265C produces in various sequence contexts, relative to wild type, to elucidate the mechanism of increased error production. We report the first complete error specificity analysis of a mutator polymerase altered in a residue not predicted to contact either the DNA or dNTP substrates.

MATERIALS AND METHODS

Reagents. Wild type rat polymerase β and mutator pol Y265C were purified as described previously (*21*). The proofreading-deficient form of the Klenow fragment of *E. coli* DNA polymerase I (Exo$^-$ Klenow) was purchased from Amersham Life Science, Inc. (Arlington Heights, IL). All restriction enzymes were supplied by Gibco BRL Life Technologies (Gaithersburg, MD) and used according to the manufacturer's protocol. The sequence of the DNA oligonucleotide used as a primer for DNA synthesis reactions is 5′-GGT ACG TAG ACG ATA TCG TC (Pharmacia, Piscataway, NJ).

HSV-tk Forward Mutational Assay. Polymerases β-wt and Y265C were used in the HSV-*tk* forward mutational assay to compare in vitro mutation frequencies, as reported previously (*21*). To ensure independence of selected mutant colonies for the mutational spectra, FT334 cells were aliquoted into multiple tubes containing VBA broth immediately after electroporation as previously described (*22*). Cultures were incubated at 37 °C for 2 h and then plated separately on selective media. To determine mutation specificities, the *Mlu*I-*Eco*RV target region of one or two mutants selected from each tube was analyzed by dideoxy DNA sequence analysis using Sequenase 2.0, according to the manufacturer's protocol (Amersham Corp., Arlington Heights, IL). Mutants selected from the same tube that contained identical mutations were not considered independent and were counted as one mutant.

Analysis of Mutation Frequencies in the HSV-tk Assay. The HSV-*tk* mutant frequency is defined as the number of colonies resistant to both 40 μM 5′-fluoro-2′-deoxyuridine (FUdR) and 50 μg/mL chloramphenicol (Cm) divided by the total number of Cm-resistant colonies. The observed mutant frequencies were adjusted for plasmids that had no alterations in the *Mlu*I-*Eco*RV region or which contained large deletions resulting in loss of this region as determined by restriction enzyme digestion. These plasmids are assumed to contain pre-existing, inactivating HSV-*tk* mutations in the gapped molecule used for hybridization. Observed mutant frequencies were also corrected for the existence of multiple mutations in order to obtain an estimated polymerase error frequency (EF_{est}) according to the following formula:

$$MF_{obs} = \sum_{n=1}^{4} (1/n)(\text{mutants with } n \text{ errors/ total sequenced mutants})(EF_{est})$$

To determine *n*, the number of independent errors (4 was the maximum observed in this study), each error within the DNA target region of a given mutant colony must be classified as (1) detectable or undetectable and (2) tandem or nontandem. Only detectable, nontandem errors are considered independent. A detectable error is defined as one that results in an FUdR-resistant phenotype in the absence of other errors. All frame shift errors are assumed to be detectable. Known detectable base substitutions are found in the HSV-*tk* database of approximately 800 sequenced mutants (K. Eckert, unpublished data). Tandem errors are defined as adjacent errors, or those occurring at sites closer than 15 nucleotides for which no evidence exists for either error occurring alone. These errors may have resulted from one complex error event by the polymerase (*23*) and are ambiguous; thus, they are excluded from specificity analyses. Conversely, errors are defined as nontandem if both occurred at sites where evidence exists for errors occurring as single or independent polymerase binding events at the same site. We assumed multiple errors spaced more than 15 nucleotides apart resulted from independent polymerase binding events, as our processivity studies show that the maximum length of DNA products synthesized per pol β binding event is 15 nucleotides under these reaction conditions (see below). EF_{est} values were calculated for each polymerase and used to calculate frequencies of specific types of errors. Differences in proportions of specific types of errors were analyzed statistically using Fisher's exact test (two-tailed) (*24*).

LacZ Opal Codon Reversion Mutation Assay. An M13mp2 *lacZ*α reversion assay was used to compare base substitution

[1] Abbreviations: Pol, polymerase; β-wt, wild type DNA polymerase β; Y265C, mutant DNA polymerase β; HSV-*tk*, herpes simplex virus type 1 thymidine kinase.

frequencies for pol β-wt and Y265C (*25*). This DNA substrate contains an opal codon at positions 87−89 which results in defective α-complementation of β-galactosidase activity. Polymerase reaction conditions to fill the 362-nucleotide gapped substrates were identical with those used in the processivity and HSV-*tk* assays, and included 0.1 pmol of DNA and either 20 pmol of β-wt or 205 pmol of Y265C. Following electroporation of in vitro synthesis products into *E. coli* strain MC1061, polymerase errors which partially restore *lacZ* activity were identified by plating on screening media as described previously (*25*). Using this method, eight possible base substitutions can be detected excluding errors at C residues and G → A base substitutions.

Processivity Reactions. Five picomoles of primer was radioactively labeled at the 5′-end using [γ-^{32}P]ATP (5000 Ci/mmol) (Amersham Corp.) and T4 polynucleotide kinase (Gibco BRL Life Technologies) according to the manufacturer's protocol, incubated with 5 pmol of M13tk3.5 ssDNA at 85 °C for 5 min, and allowed to cool to room temperature. Hybridization reaction mixtures were purified using G-50 Sephadex Quick Spin columns (Boehringer Mannheim Corp., Indianapolis, IN) and Microcon-30 ultrafiltration units (Amicon Inc., Beverly, MA). For each hybrid, a control for the percent hybridization of primer/template substrate was performed by incubating 150 fmol of Klenow Exo$^-$ and 250 fmol of primed M13tk3.5 ssDNA substrate in buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM dNTPs for 60 min at 37 °C. For the processivity reactions, 500 fmol of oligonucleotide-primed ssDNA was preincubated at 37 °C for 3 min, using reaction conditions identical to those of the HSV-*tk* assay. Synthesis reactions were initiated by the addition of polymerase, in amounts indicated in the figure legends. Aliquots were removed at various times and added to an equal volume of stop dye (99% formamide, 5 mM EDTA, 1 mg/mL xylene cylanol, and 1 mg/mL bromophenol blue). DNA reaction products were separated on a 12% denaturing polyacrylamide gel, using a DNA sequencing ladder as a marker. Quantitation of DNA reaction products was performed using a Molecular Dynamics Phosphorimager (Sunnyvale, CA). The amount of DNA in the primer band was corrected for the percent hybridization of radiolabeled primer to templates. The degree of DNA synthesis for each polymerase was defined for each reaction as the percent primer/template extension, calculated as the amount of extended primer/template molecules divided by the total amount of primer/template molecules in the reaction (extended + unextended).

RESULTS

Specificity of Polymerases Y265C and β-wt in the HSV-tk Forward Mutation Assay. To elucidate the mechanism of the Y265C mutator polymerase, we have compared the error types produced by Y265C with those produced by β-wt under identical reaction conditions. HSV-*tk* mutants were produced during in vitro primer extension DNA synthesis using an oligonucleotide-primed ssDNA template and were selected in *E. coli*, as previously described (*21, 22*). Table 1 summarizes the results based on DNA sequence analysis of 79 mutants from one Y265C reaction and 86 mutants collected from three β-wt reactions. The specific sequence changes of the mutants are presented in Figures 1 and 2. The observed mutant frequencies are 14×10^{-4} and 440×10^{-4} for β-wt and Y265C, respectively. We observed a 13-fold increase in the frequency of Y265C HSV-*tk* mutants containing only one error within the 203-base pair target region relative to β-wt (Table 1). Moreover, the frequency of mutants containing two or more errors in the DNA target sequence is markedly increased (530-fold) for Y265C over β-wt. This increase in the frequency of multiple errors was reproduced in an independent synthesis reaction using a different preparation of Y265C, where DNA sequence analysis of 28 mutants demonstrated a frequency of 240×10^{-4} for single errors and 320×10^{-4} for multiple errors.

Table 1: Error Specificity by Class of Y265C and β-wt in the HSV-*tk* Forward Assay

	frequency × 10^{-4} (number observed)		
error class	Y265C	β-wt	Y265C/β-wt
MF_{obs}[a]	440 (79)	14 (86)	31
single	180 (33)	14 (83)	13
multiple	260 (46)	0.49 (3)	530
EF_{est}[b]	470	14	34
base substitution	74	3.2	23
frame shift	330	10	33

[a] MF_{obs} is the average observed mutation frequency from one Y265C reaction and three β-wt reactions. [b] EF_{est} is the estimated polymerase error frequency (see Materials and Methods) which corrects the observed mutation frequency for multiple errors. EF_{est} is used to calculate the error frequency of detectable base substitution and frame shift errors.

To compare accurately the frequencies of specific types of polymerase errors for Y265C and β-wt, the observed mutant frequency was corrected for the existence of multiple errors (see Materials and Methods). As shown previously, each FUdR-resistant colony reflects one detectable polymerase error in the HSV-*tk* target (*22*); therefore, mutants that contain more than one detectable error within the target region result in an underestimation of the true polymerase error frequency. For example, two phenotypically detectable errors occurring in the target region of one mutant colony would have resulted in two mutant colonies had the errors occurred on separate DNA molecules. The estimated polymerase error frequency for Y265C is 470×10^{-4} and for β-wt is 14×10^{-4}, a 34-fold difference (Table 1). A comparable increase was observed for frame shift and base substitution errors; therefore, an increase in both types of errors contribute nearly equally to the observed mutator phenotype. Similar to pol β-wt, the Y265C mutator produces a higher proportion of frame shift errors than base substitution errors. Moreover, the characteristic pattern of error-prone hot spots in homopolymeric template sequences observed in the error spectrum of β-wt is maintained in the Y265C spectrum (Figure 1), indicating that the mutator Y265C retains some error specificity features of β-wt.

Effect of Y265C Substitution on Misalignment-Mediated Errors. The molecular mechanism for error production in homonucleotide repeat sequences involves polymerase extension synthesis from misaligned primer/template DNA structures, which can result in two distinct frame shift errors (*26*). If the misalignment results in extrahelical template bases, deletion errors result, whereas extrahelical bases in the primer strand result in base addition errors. An overall increase in the frequency of frame shift events (Table 1) was measured for Y265C compared to that for β-wt, the majority of which occur in homonucleotide or dinucleotide repeats (Figure 1).

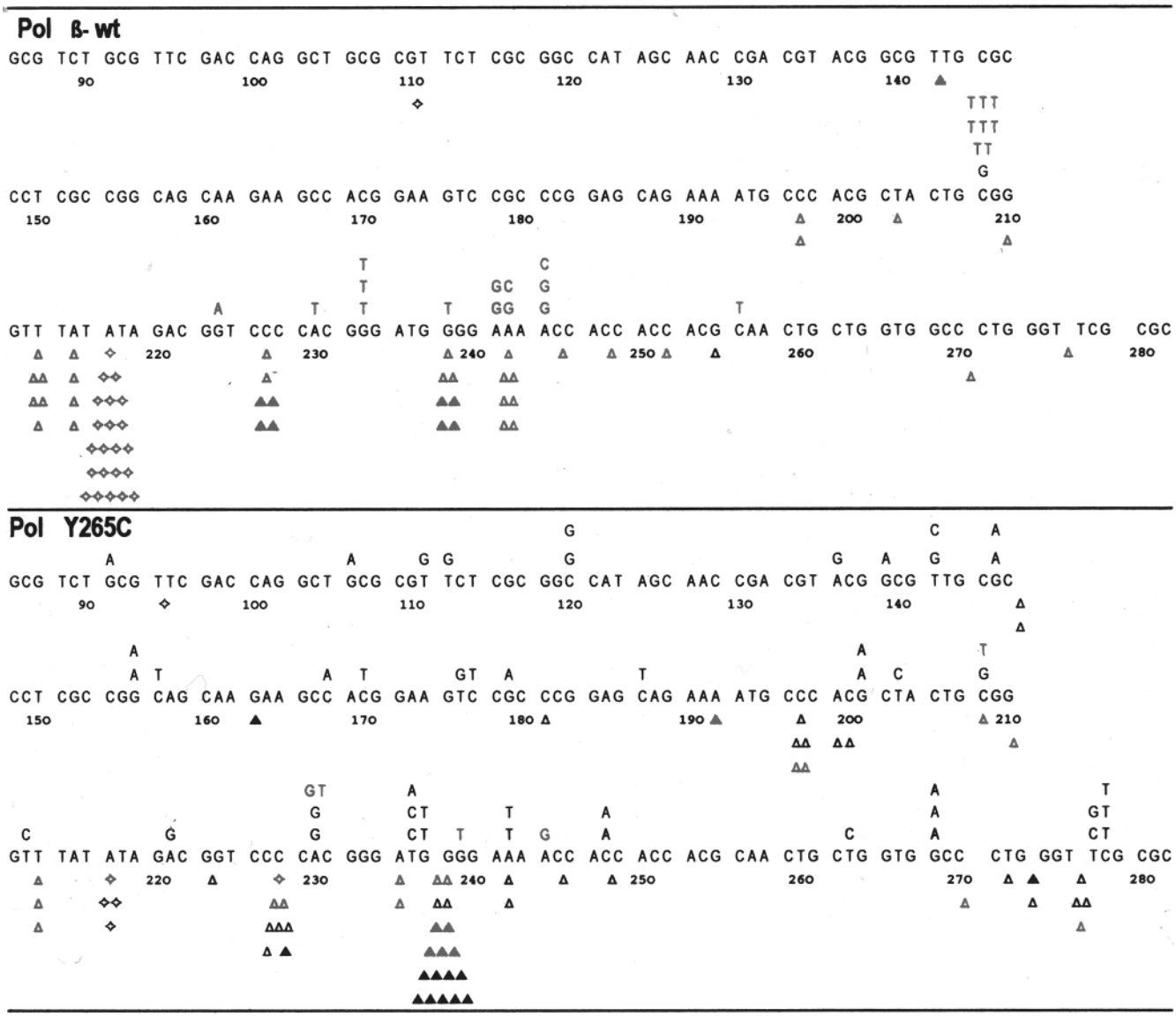

FIGURE 1: HSV-*tk* forward mutational specificity of polymerases β-wt and Y265C. The DNA template shown is the *Mlu*I-*Eco*RV mutational target containing the ATP binding site of the HSV-*tk* gene. Letters above the line indicate base substitutions, and symbols below the line represent frame shift mutations: one-base deletions (△), one-base additions (▲), and two-base deletions (◇). All single change mutational events are indicated in red. Not shown on the spectra are β-wt deletions (27 base pairs between 242 and 268, 9 base pairs between 221 and 229, and 29 base pairs between 241 and 269) and Y265C deletions (28 base pairs between positions 241 and 268 and 28 base pairs between positions 120 and 147, as part of a multiple error, Figure 2). Tandem multiple errors (Figure 2) were excluded.

We determined that, for both Y265C and β-wt, one-base frame shift error rates increased 7-fold as the repeat length increased from two to three nucleotides and 4-fold as the length increased from three to four nucleotides (Table 2). Therefore, while Y265C retains a one-base frame shift error rate versus repeat length dependence similar to that of β-wt, this mutator demonstrates an increased error rate at all repeat lengths (~50-fold), relative to that of β-wt.

The one-base deletion frequency represents the largest frame shift class for both polymerases (Figure 3). The 39-fold increase in the frequency of these errors for Y265C, relative to that of β-wt, is similar to the 33-fold increase observed for total frame shift errors (Table 1). In contrast, the two-base deletion frequency is increased by only 8.6-fold, whereas the one-base addition frequency is increased 72-fold for Y265C, relative to that of β-wt (Figure 3). These differences in observed frequency increase for Y265C suggest the mutator may be markedly more error-prone for primer slippage events (resulting in one-base additions), relative to other frame shift mechanisms and to that of β-wt. Alternatively, sequence context effects may explain the difference in mutator effect for two-base deletions and one-base additions, whereby alterations in polymerase−primer/template interactions result in different error-prone hot spots. For example, the majority of two-base deletions occur at the $(TA)_3$ dinucleotide repeat at positions 214−219 (Figure 1), a hot spot for two-base deletions observed previously for an independent preparation of pol β-wt (*22*). This is the only site where a large difference is observed in the proportions of two-base deletions produced by each polymerase. While Y265C does display an increased frequency of two-base deletions (4-fold) at this site relative to that of β-wt (Figure 3), the proportion of these errors relative to total frame shifts is significantly lower for Y265C ($^6/_{63}$) than for β-wt ($^{23}/_{63}$) ($p = 0.0001$). Another hot spot for Y265C is the $(G)_4$ sequence at positions 237−240 where a large majority of

Site	Error	Site	Error
I. Pol Y265C			
A. Double Errors			
106	G→A	163	▲G
111	T→G	237-240	▲G
120	C→G	226-229	ΔC
136	A→G	237-240	▲G
139	G→A	237-240	▲G
146	G→A	200	ΔC
146	G→A	223-224	ΔG
146-149	ΔC	236	T→A*
168	C→A	230	A→G*
170	C→T	248-249	ΔC
177	C→T	237-240	ΔG
179	G→A	226-229	ΔC
180-182	ΔC	245-246	ΔC
196-198	ΔC	120-147	Δ28 bp
196-198	ΔC	278	C→T
196-198	ΔC	273-275	▲G
199	ΔA	276-277	ΔT
201	G→A	214-219	◇TA
208	C→G	214-219	◇TA
212	T→C	230	A→G*
226-229	ΔC	268	G→A
226-229	ΔC	277	T→G*
237-240	▲G	242	A→T*
237-240	ΔG	277	T→C
237-240	▲G	278	C→T
241-244	ΔA	248	C→A
242	A→T*	268	G→A
248	C→A	276-277	ΔT
273-275	ΔG	278	C→T
B. Tandem or near tandem doubles			
226-229	ΔC	228-229	C→T*
237-240	▲G	244-252	-(ACC)
237-240	ΔG	244-252	-(ACC)
241	A→G*	244-252	-(ACC)
242	A→G*	247	A→T
245-246	C→T*	245-246	ΔC
C. Triple Mutants			
91	G→A	147-149	ΔC
276-277	ΔT		
94-95	◇T	156	G→A
236	T→C		
100	***C→T***	***105***	***ΔT***
272	ΔT		
143	***T→C***	***146***	***G→A***
237-240	▲G		
156	G→A	203	T→C*
236	T→C		
157	C→T	187	C→T
263	T→C*		
176	T→G	201	G→A
226-229	▲C		
200	***ΔC***	***206***	***ΔT***
237	G→T		
D. Quadruple mutants			
106-109	***◇GC***	***111***	***T→G***
221	A→G*	237	G→T
112	T→G	142	T→G
214-219	◇TA	268	G→A
120	C→G	142	T→C
200	***ΔC***	***206***	***ΔT***
II. Pol β-wt Double mutants			
208	C→G	214-219	◇TA
110-111	◇GT	255	ΔG
245	***C→T***	***246***	***C→T***

FIGURE 2: List of Y265C and β-wt HSV-*tk* mutants containing multiple errors. Italicized changes denote tandem or near tandem errors. Detectable base substititions are indicated by an asterisk. Frame shifts: one-base deletions (△), one-base additions (▲), and two-base deletions (◇). The T → C base substitution at position 263 is assumed to be phenotypically detectable alone because it results in an amino acid change from leucine to proline. In addition, the other two errors in this mutant result in a TAG nonsense condon which is suppresible in the FT334 strain.

Table 2: Error Rates of One-Base Frame Shifts as a Function of Repeat Length in the HSV-*tk* Forward Assay

no. of nucleotides in the repeat	error rate[a] × 10^{-5} (number observed)		
	Y265C	β-wt	Y265C/β-wt
1	1.8 (7)	0.047 (6)	39
2	14 (7)	0.29 (5)	48
3	98 (15)	2.0 (10)	49
4	370 (28)	7.9 (20)	47
frame shift EF	3300 (63)	100 (63)	34

[a] Error rates were calculated by multiplying the total frame shift error frequency (EF; see Table 1) by the proportion of one-base frame shifts at each repeat length per total frame shifts and then dividing by the number of occurrences of each repeat length in the target sequence.

the one-base additions occur. Interestingly, Y265C does not demonstrate a high prevalence of one-base additions elsewhere in the target sequence (Figure 1). β-wt also makes one-base additions at the $(G)_4$ site, but the frequency of Y265C-produced additions here is much higher (86-fold, Figure 3), and the difference in proportion of these errors, relative to total frame shifts, between the polymerases is significant ($p = 0.02$). On the basis of these data, the differences observed in the proportion of one-base additions and two-base deletions between Y265C and β-wt most likely reflects sequence-dependent differences in template interactions by the two polymerases. Thus, we do not believe Y265C is a mutator specifically for the primer slippage events which result in one-base additions, but rather, Y265C is a general mutator for misalignment-initiated frame shifts.

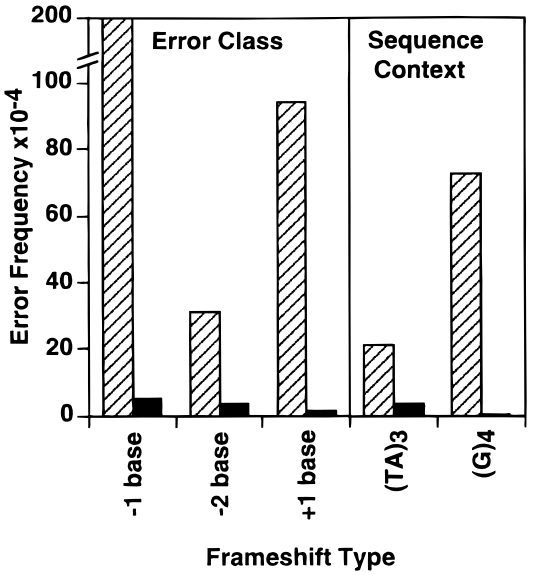

FIGURE 3: Frame shift error specificity of polymerases Y265C and β-wt. Error frequency values are calculated using the estimated polymerase error frequency, EF_{est}. This analysis includes 63 independent frame shifts for each polymerase and excludes frame shifts that occurred as a tandem error event. Values in the sequence context category represent the frequency of two-base deletions at the $(TA)_3$ repeat, positions 214−219, and one-base additions at the $(G)_4$ repeat, positions 237−240 in the HSV-*tk* gene: hatched bars, Y265C; and solid bars, β-wt.

Effect of Y265C Substitution on Mispairing-Mediated Errors. The molecular mechanism for error production by direct polymerase miscoding is distinct from that of primer/template misalignments; therefore, we analyzed the effect of the mutator Y265C on this class of mutations. The HSV-*tk* forward assay was used to determine the types and proportion of base substitution errors Y265C produces, as all 12 possible mispair errors can be detected in this target. As shown in Table 1, Y265C has a 23-fold higher base substitution frequency than β-wt, comparing only those base substitutions known to result in a detectable HSV-*tk* mutant phenotype. However, the majority of Y265C-produced base substitutions ($^{47}/_{52}$) occur as multiple errors (Figures 1 and 2). The phenotypic consequences of these errors are unknown, as the HSV-*tk* gene is not fully saturated for all possible inactivating base substitution mutations, precluding direct comparison with the β-wt error spectrum. Therefore, a *lacZ* opal codon reversion assay, which focuses on base substitution errors at three template positions during gap-filling synthesis, was used to compare Y265C and β-wt base substitution specificities. In this assay, the base substitution frequency of Y265C is 46-fold higher than that observed for β-wt (Table 3).

The ranking of specific base substitution errors for Y265C at the HSV-*tk* gene are presented in column 3 of Table 3 as proportions of total observed base substitutions. The transition base substitutions represent the largest class (65%) and are ranked in decreasing order of frequency as follows: G·dTMP > C·dAMP ≥ T·dGMP ≥ A·dCMP. The next largest class observed were pyrimidine·pyrimidine (pyr·pyr) mispairs (23%) followed by purine·purine (pur·pur) mispairs (12%). All four possible pyr·pyr mispair errors were detected for Y265C; however, only two of four possible pur·pur mispairs were detected (A·dAMP and G·dAMP).

Table 3: Base Substitution Error Specificity of Polymerases Y265C and β-wt

base substitution class	mispair	number observed (proportion) HSV-*tk* gene Y265C	*lacZ* gene[b] Y265C	*lacZ* gene[b] β-wt
T → A	T·dTMP	1 (0.019)	3 (0.020)	1 (0.0087)
T → G	T·dCMP	5 (0.10)	16 (0.10)	0 (<0.0087)
T → C	T·dGMP	7 (0.13)	66 (0.43)	4 (0.035)
A → T	A·dAMP	3 (0.058)	4 (0.026)	10 (0.087)
A → G	A·dCMP	6 (0.12)	56 (0.37)	94 (0.82)
A → C	A·dGMP	0 (<0.019)	1 (0.0065)	0 (<0.0087)
G → T	G·dAMP	3 (0.058)	6 (0.039)	3 (0.026)
G → A	G·dTMP	13 (0.25)	ND	ND
G → C	G·dGMP	0 (<0.019)	1 (0.0065)	3 (0.026)
C → T	C·dAMP	8 (0.15)	ND	ND
C → A	C·dTMP	3 (0.058)	ND	ND
C → G	C·dCMP	3 (0.058)	ND	ND
overall base substitution error frequency $\times 10^{-4}$		74[a]	87 ± 19	1.9 ± 0.1

[a] The error frequency for the HSV-*tk* gene was calculated using the EF_{est} and only detectable base substitutions. [b] For the LacZ reversion assay, total mutants included 153 for Y265C and 115 for β-wt. The error rate per nucelotide (*25*) can be calculated with the formula (error frequency × proportion)/0.6. ND = not detectable.

The base substitution error specificity at the *lacZ* gene opal codon of Y265C is compared to β-wt in columns 4 and 5 of Table 3. Y265C has an increased frequency of all detectable base substitution types, relative to β-wt. The T·dGMP mispair occurs in the highest proportion at the *lacZ* gene and at a frequency 580-fold higher than that of β-wt (Table 3). The transition to transversion base substitution ratio is slightly higher for β-wt (5.7) than for Y265C (4.0) (Table 3). The change in this ratio is due to a significantly higher proportion of Y265C-produced transversion errors resulting from pyr·pyr mispairs (0.12), relative to that of β-wt (0.01) ($p = 0.0002$). In contrast, there is no significant difference in proportion of errors resulting from purine·purine (pur·pur) mispairs between the two polymerases (Y265C, 0.08; β-wt, 0.14). In summary, whereas the Y265C mutant frequency is generally increased for all base substitution classes, the magnitude of increase over that of β-wt is highest for T·dGMP and pyr·pyr mispairs.

Effect of Y265C Substitution on Processivity. We established the processivity of the mutator polymerase to determine whether the multiple errors produced by Y265C resulted from a single polymerase binding event during one round of DNA synthesis or from several independent polymerase binding events. Measuring processivity requires the limitation of primer extension to one round of synthesis and is influenced by substrate and reaction conditions (*27*). Due to the 20-fold differences in the k_{cat} for the two forms of pol β analyzed (*21*), we chose to conduct processivity analyses using the template excess method described by Bambara et al. (*27*). We used conditions of nearly identical amounts of DNA synthesis for each polymerase and a reaction buffer composition and oligonucleotide-primed circular ssDNA substrate identical with that used in the mutational analyses.

The magnitude of template excess necessary to efficiently prevent polymerase re-binding to the previously extended DNA molecule was determined empirically for each polymerase by ensuring that the reaction product distribution was independent of enzyme concentration and time (*27*). Reaction products were quantitated for various amounts of Y265C and β-wt at a constant substrate concentration, and the population distribution of product molecules was determined. As the enzyme amount was decreased from 260 fmol of Y265C and from 21 fmol of β-wt, the product distribution remained constant (Figure 4A). A representative autoradiogram (Figure 4A) shows DNA synthesis products for reaction mixtures containing 100 and 53 fmol of Y265C (lanes 1−2) and 10 and 5.2 fmol of β-wt (lanes 3−4). For these reactions, the product distribution is independent of enzyme concentration, indicating products represent one cycle of DNA synthesis. The product distribution is also independent of time as shown by the time course reactions (Figure 4B). The total percent of primer/template extended also varies little up to a 60 min reaction time. Therefore, we believe that upon polymerase dissociation, the majority of the enzymes may bind nonproductively to single-stranded regions of the primer/template via the polymerase 8 kDa domain, which demonstrates high affinity for ssDNA (*28*).

Comparing pol Y265C and β-wt one-cycle synthesis products, we observe that both polymerases exhibit generally distributive synthesis when utilizing an oligo-primed ssDNA template. When DNA synthesis is initiated from primer 282 (Figure 4B), molecules up to nine nucleotides long for Y265C and eight nucleotides for β-wt are visible, comparing reactions of equivalent DNA synthesis amounts (≈15%). However, a large proportion, 45 ± 1 and 44 ± 2%, of the primer/templates were extended by only one nucleotide for β-wt and Y265C polymerases, respectively, while the other half continued to incorporate a few additional nucleotides. Thus, the majority of Y265C enzymes synthesize DNA in a near distributive manner, similar to β-wt, indicating that the multiple errors observed during Y265C DNA synthesis result from several polymerase·DNA binding events.

DISCUSSION

In this study, we determined the error specificity of the catalytically active polymerase β mutator, Y265C, relative to β-wt, to elucidate the critical role amino acid tyrosine 265 plays in the fidelity of pol β DNA synthesis. Our results demonstrate that polymerase Y265C is a mutator for frame shifts in homonucleotide repeat sequences as well as for base substitution errors in heteronucleotide sequences during in

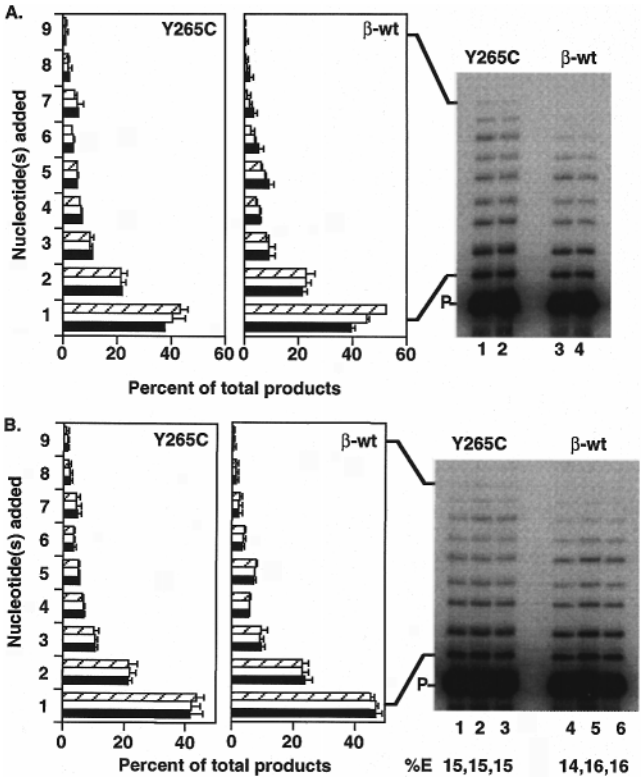

FIGURE 4: Comparison of polymerases Y265C and β-wt during one cycle of DNA synthesis products at the HSV-*tk* gene. (A) DNA synthesis distribution as a function of the template:enzyme ratio. (Left panel) Population distribution of primer extension products for various amounts of Y265C and β-wt and a constant amount of ssDNA primer/template (500 fmol). Y265C amounts include 260 fmol (filled bars), 100 fmol (open bars), and 53 fmol (hatched bars). β-wt amounts include 21 fmol (filled bars), 10 fmol (open bars), and 5.2 fmol (hatched bars). Values for 100 and 53 fmol of Y265C and 21 fmol of β-wt represent averages and standard deviations from three independent reactions, and values for 10 and 5.2 fmol of β-wt represent averages and standard deviations from two independent reactions. (Right panel) Autoradiograms of primer extension products separated on a 12% denaturing polyacrylamide gel. Mixtures from 60 min reactions contained 500 fmol of oligonucleotide-primed ssDNA template of either Y265C [100 fmol (lane 1) and 53 fmol (lane 2)] or β-wt [10 fmol (lane 3) and 5.2 fmol (lane 4)]. (B) DNA synthesis distribution as a function of time. (Left panel) Population distribution of primer extension products from reactions including 500 fmol of primed ssDNA template and either 53 fmol of Y265C or 10 fmol of β-wt, terminated at 15 min (filled bars), 30 min (open bars), and 60 min (hatched bars). Values for Y265C and β-wt represent averages and standard deviations from three and two independent reactions, respectively. (Right panel) Autoradiograms of primer extension products separated on a 12% denaturing polyacrylamide gel. Time course reactions are 15, 30, and 60 min for Y265C (lanes 1−3) and for β-wt (lanes 4−6). %*E* values represent the percentage of primers extended for each reaction.

vitro primer extension synthesis. The mechanisms described for the production of these error types are distinctly different: primer/template misalignment and base misincorporation, respectively. In addition, Y265C displays a 530-fold increase in multiple errors within the small target region examined, relative to that of β-wt. Localization of tyrosine 265 in the X-ray crystallographic structure (*10*) suggests that this residue may play a role in mediating the polymerase conformational change predicted to occur after dNTP binding but prior to chemistry. The following discussion will interpret the error specificity results of the Y265C mutator in conjunction with the X-ray crystallographic studies, and will propose a role for Y265 in the fidelity of polymerase β DNA synthesis.

Polymerase Y265C is the first well-characterized mutator polymerase that demonstrates a dramatic increase in the frequency of multiple errors within a small target region, relative to β-wt (Table 1). This finding is quite remarkable, since Y265C is altered in an amino acid that is not predicted to interact with the DNA and dNTP substrates (*10*). Therefore, we examined the effect of the Y265C alteration on the processivity of DNA synthesis in order to determine whether the multiple mutations could have been produced during a single polymerase binding event. The processivity of pol β has been shown to vary significantly depending on the activating metal cation, dNTP substrate concentration, and DNA primer/template structure (*29*−*31*). Using the same primer/template and buffer conditions (1 mM dNTPs) that were used in the HSV-*tk* forward mutational assay, we demonstrate that the majority of both Y265C and β-wt enzymes synthesize DNA in a near distributive manner, adding one or two nucleotides per association (Figure 4). This laddering pattern has been described previously for pol β using similar primer/template substrates (*30*). In our system, Y265C retains the distributive nature of DNA synthesis that is characteristic of β-wt, and is not a processivity mutant. Therefore, for β-wt and Y265C, many polymerase binding events are necessary to complete synthesis of the mutational target. We interpret the increase in multiple errors seen in the Y265C spectrum as being due to an increased Y265C error rate per binding cycle. An increased frequency of multiple errors relative to β-wt may be a characteristic of catalytically active pol β mutators, given the requirement of multiple binding events to synthesize across an entire gene.

The Y265C mutator is unique in that it displays a nearly equal increase in frequency of both base substitution and frame shift errors, relative to wild type, which result from errors initiated by base mispairing and primer/template misalignment, respectively (Figure 1 and Table 1). The one-base frame shift error rates for both Y265C and β-wt increase as the length of the homonucleotide repeat increases (Table 2), as observed previously for β-wt and other DNA polymerases (*26*, *32*). This relationship is consistent with a frame shift error mechanism initiated by primer/template misalignment, since as the repeat length increases, the number of correct base pairs that could potentially stabilize the misaligned primer/template also increases (*26*). In addition, Y265C displays a mutator phenotype for base misincorporation-initiated errors at two different mutational targets, the HSV-*tk* gene and the *lacZ* gene (Table 3). In this respect, pol Y265C differs from previously identified mutator polymerases which demonstrate an increase in the frequency of errors initiated by one mechanism or the other (*33*). For example, mutator forms of HIV-1 RT that are more error-prone for misalignment-initiated errors contain an alteration in the proposed thumb domain, suggesting that this domain may be important for selecting against primer/template misalignments (*13*). Alternatively, several mutant polymerases have been described that are altered in an amino acid predicted to interact with the substrate at the active site. Those which are mutators demonstrate an increase specifically in misincorporation-mediated base substitutions (*6*, *15*,

16, *20*, *34*). The alteration in Y265C affects a distinctly different error discrimination mechanism, one that selects nearly equally against both misalignment and misincorporation premutational intermediates.

Both β-wt and Y265C demonstrate the ability to form and extend mispairs, and the observed increase in base substitution frequency for Y265C reflects reduced fidelity at either or both steps. The ranking of base substitution proportions for Y265C observed at the HSV-*tk* gene is very similar to that at the *lacZ* gene. The most prevalent base substitution observed for Y265C at the HSV-*tk* gene resulted from G·T mispairs. Unfortunately, none of these occurred at HSV-*tk* sites known unequivocally to be phenotypically detectable, and this mispair is not detectable in the *lacZ* opal codon reversion assay, precluding comparison with the β-wt spectrum. However, the reciprocal error, T·G, was the most prevalent Y265C mispair detected in the reversion assay and the third most prevalent in the forward assay (Table 3). The T·G and G·T mispairs are stable, wobble base pairs. Structural studies indicate that these mispairs do not significantly alter the global DNA structure and are potentially stabilized by hydrogen bonding with water (*35*). The observed increase in frequency of base substitution errors occurring through T·G and possibly G·T mispairs by the mutator may result from stabilization in the Y265C polymerase binding pocket by hydrogen bonding with water molecules that are normally excluded from the wild type binding pocket (*36*). We also observed the production of a significantly higher proportion of base substitutions resulting from pyrimidine·pyrimidine mispairs by Y265C compared to wild type at the *lacZ* target (Table 3). These may result from stabilization of the geometrically small pyrimidine·pyrimidine mispair in the binding pocket of the pol Y265C active site, perhaps again by hydrogen bonding with water molecules that are normally excluded from the wild type binding pocket. These data are consistent with the hypothesis that the binding pocket of Y265C may be in an aberrant conformation.

The amino acid change for polymerase Y265C is located in a hinge region that connects the two subdomains. On the basis of X-ray crystallographic analyses, Pelletier et al. (*10*) observed a difference in the thumb subdomain position between the binary complex (DNA−enzyme) and the ternary complex (DNA−enzyme−ddCTP). This movement, whereby the thumb closes over the substrate, is predicted to result from changes in four torsion angles of amino acids 263, 264, and 265 (*10*) and may be similar to the polymerase conformation change that is predicted to occur in pol β prior to chemistry (*5*). A conformational change has been postulated to play an important role in the fidelity of DNA synthesis by imposing an induced fit selectivity (*4*). By bringing the template and incoming base into correct geometrical alignment for phosphodiester bond formation, the configuration of the closed active site discriminates against errors. The substitution of cysteine for tyrosine at position 265 may affect pol β fidelity directly, through changes in this thumb movement, or indirectly, through changes in the positioning of another residue critical for error discrimination. We favor the hypothesis that the amino acid change in residue 265 of polymerase β affects the conformational change that mediates induced fit selectivity and consequently affects error discrimination by the polymerase. Y265C may form an aberrant or less stable closed conformation or, alternatively, may be completely defective in changing conformation. As the hinge region is lined on either side by hydrophobic residues, the introduction of a polar side chain (cysteine) might destabilize or alter the structure of this region (*10*). A defect in the conformational change formed by the Y265C polymerase may loosen the constraints normally imposed on newly formed base pairs, thus allowing efficient DNA synthesis utilizing mispaired and misaligned intermediates. The increase in both frame shifts due to misalignment and base substitutions due to misincorporation for pol Y265C is consistent with such a model.

Our in vitro studies utilizing primed ssDNA templates allowed for a detailed, structure−function comparative analysis of Y265C processivity and fidelity relative to those of β-wt. The in vivo pol β environment will undoubtedly differ with regard to the DNA substrate form (e.g. chromatin) and the protein form (e.g. multiprotein repair complex), and this may affect polymerase β error specificity. Remarkably, however, we have observed a similar magnitude of the Y265C mutator effect (about 30-fold) in vitro and during the in vivo genetic screen in *E. coli* (*21*). Kinetic studies have shown that the Y265C mutator polymerase remains catalytically active and thus has the potential for contributing to genomic instability if the mutator effect is expressed in vivo. In eukaryotic mammalian cells, evidence suggests pol β plays a role in both short-patch and long-patch base excision repair pathways (*37*−*41*), and possibly in DNA replication (*42*, *43*). Interestingly, studies have shown that the pol β gene is mutated in several human tumors (*44*−*46*); however, the functional consequences of such mutations are unknown. Given the role of pol β in DNA repair synthesis, a mutator form such as Y265C expressed in vivo might have considerable effects on the integrity of the genome.

ACKNOWLEDGMENT

We thank Guang Yan, Margaret Yoon, and Stacy Washington for providing technical support and Drs. Thomas A. Kunkel, Lawrence A. Loeb, Kenneth Johnson, and Ross Shiman for critical reading of the manuscript. We gratefully acknowledge the generous contributions made to The Jake Gittlen Cancer Research Institute.

REFERENCES

1. Kuchta, R. D., Benkovic, P., and Benkovic, S. J. (1988) *Biochemistry 27*, 6716−6725.
2. Dahlberg, M. G., and Benkovic, S. J. (1991) *Biochemistry 30*, 4835−4843.
3. Patel, S. S., Wong, I., and Johnson, K. A. (1991) *Biochemistry 30*, 511−525.
4. Johnson, K. A. (1993) *Annu. Rev. Biochem. 62*, 685−713.
5. Werneburg, B. G., Ahn, J., Zhong, X., Hondal, R. J., Kraynov, V. S., and Tsai, M.-D. (1996) *Biochemistry 35*, 7041−7050.
6. Ahn, J., Werneburg, B. G., and Tsai, M.-D. (1997) *Biochemistry 36*, 1100−1107.
7. Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., and Steitz, T. A. (1985) *Nature 313*, 762−766.
8. Kim, Y., Eom, S. H., Wang, J., Lee, D. S., Suh, S. W., and Steitz, T. A. (1995) *Nature 376*, 612−616.
9. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) *Science 256*, 1783−1790.

10. Pelletier, H., Sawaya, M. R., Wolfle, W., Wilson, S. H., and Kraut, J. (1996) *Biochemistry 35*, 12742−12761.
11. Wang, J., Sattar, A. K. M. A., Wang, C. C., Karam, J. D., Konigsberg, W. H., and Steitz, T. A. (1997) *Cell 89*, 1087−1099.
12. Joyce, C. M., and Steitz, T. A. (1994) *Annu. Rev. Biochem. 63*, 777−822.
13. Bebenek, K., Beard, W. A., Casas-Finet, J. R., Kim, H.-R., Darden, T. A., Wilson, S. H., and Kunkel, T. A. (1995) *J. Biol. Chem. 270*, 19516−19523.
14. Minnick, D. T., Astatke, M., Joyce, C. M., and Kunkel, T. A. (1996) *J. Biol. Chem. 271*, 24954−24961.
15. Carroll, S. S., Cowart, M., and Benkovic, S. J. (1991) *Biochemistry 30*, 804−813.
16. Bell, J. B., Eckert, K. A., Joyce, C. M., and Kunkel, T. A. (1997) *J. Biol. Chem. 272*, 7345−7351.
17. Dong, Q., Copeland, W. C., and Wang, T. S.-F. (1993) *J. Biol. Chem. 268*, 24163−24174.
18. Martin-Hernandez, A. M., Domingo, E., and Menendez-Arias, L. (1996) *EMBO J. 15*, 4434−4442.
19. Pandey, V. N., Kaushik, N., Rege, N., Sarafianos, S. G., Yadav, P. N. S., and Modak, M. J. (1996) *Biochemistry 35*, 2168−2179.
20. Beard, W. A., Osheroff, W. P., Prasad, R., Sawaya, M. R., Jaju, M., Wood, T. G., Kraut, J., Kunkel, T. A., and Wilson, S. H. (1996) *J. Biol. Chem. 271*, 12141−12144.
21. 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) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1321−1326.
22. Eckert, K. A., Hile, S. E., and Vargo, P. L. (1997) *Nucleic Acids Res. 25*, 1450−1457.
23. Ripley, L. S. (1990) *Annu. Rev. Genet. 24*, 189−213.
24. Rosner, B. (1995) *Fundamentals of Biostatistics*, Wadsworth Publishing Co., Belmont, CA.
25. Bebenek, K., and Kunkel, T. A. (1995) *Methods Enzymol. 262*, 217−232.
26. Kunkel, T. A. (1990) *Biochemistry 29*, 8003−8011.
27. Bambara, R. A., Fay, P. J., and Mallaber, L. M. (1995) *Methods Enzymol. 262*, 270−280.
28. Kumar, A., Widen, S. G., Williams, K. R., Kedar, P., Karpel, R. L., and Wilson, S. H. (1990) *J. Biol. Chem. 265*, 2124−2131.
29. Tanabe, K., Bohn, E. W., and Wilson, S. H. (1979) *Biochemistry 18*, 3401−3406.
30. Abbotts, J., SenGupta, D. N., Zmudzka, B., Widen, S. G., Notario, V., and Wilson, S. H. (1988) *Biochemistry 27*, 901−909.
31. Singhal, R. K., and Wilson, S. H. (1993) *J. Biol. Chem. 268*, 15906−15911.
32. Kunkel, T. A. (1985) *J. Biol. Chem. 260*, 5787−5796.
33. Minnick, D. T., and Kunkel, T. A. (1996) *Cancer Surv. 28*, 3−19.
34. Kraynov, V. S., Werneburg, B. G., Zhong, X., Lee, H., Ahn, J., and Tsai, M.-D. (1997) *Biochem. J. 323*, 103−111.
35. Kennard, O. (1987) in *Nucleic Acids and Molecular Biology* (Eckstein, F., and Lilley, D. M. J., Eds.) Springer-Verlag, Berlin, Germany.
36. Petruska, J., Sowers, L. C., and Goodman, M. F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1559−1562.
37. Wiebauer, K., and Jiricny, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5842−5845.
38. Oda, N., Saxena, J. K., Jenkins, T. M., Prasad, R., Wilson, S. H., and Ackerman, E. J. (1996) *J. Biol. Chem. 271*, 13816−13820.
39. Singhal, R. K., Prasad, R., and Wilson, S. H. (1995) *J. Biol. Chem. 270*, 949−957.
40. Sobol, R. W., Horton, J. K., Kühn, R., Gu, H., Singhal, R. K., Prasad, R., Rajewsky, K., and Wilson, S. H. (1996) *Nature 379*, 183−186.
41. Klungland, A., and Lindahl, T. (1997) *EMBO J. 16*, 3341−3348.
42. Jenkins, T. M., Saxena, J. K., Kumar, A., Wilson, S. A., and Ackerman, E. J. (1992) *Science 258*, 475−478.
43. Sweasy, J. B., and Leob, L. A. (1991) *J. Biol. Chem. 267*, 1407−1410.
44. Wang, L., Patel, U., Ghosh, L., and Banerjee, S. (1992) *Cancer Res. 52*, 4824−4827.
45. Matsuzaki, J., Dobashi, Y., Miyamoto, H., Ikeda, I., Fujinami, K., Shuin, T., and Kubota, Y. (1996) *Mol. Carcinog. 15*, 38−43.
46. Dobashi, Y., Shuin, T., Tsuruga, H., Uemura, H., Torigoe, S., and Kubota, Y. (1994) *Cancer Res. 54*, 2827−2829.

BI9722711