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Amino Acid Substitutions at Conserved Tyrosine 52 Alter Fidelity and Bypass Efficiency of Human DNA Polymerase η*

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DNA polymerase η (Polη) is a member of a new class of DNA polymerases that is able to copy DNA containing damaged nucleotides. These polymerases are highly error-prone during copying of unaltered DNA templates. We analyzed the relationship between bypass efficiency and fidelity of DNA synthesis by introducing substitutions for Tyr-52, a highly conserved amino acid, within the human DNA polymerase η (hPolη) finger domain. Most substitutions for Tyr-52 caused reduction in bypass of UV-associated damage, measured by the ability to rescue the viability of UV-sensitive yeast cells at a high UV dose. For most mutants, the reduction in bypass ability paralleled the reduction in polymerization activity. Interestingly, the hPolη Y52E mutant exhibited a greater reduction in bypass efficiency than polymerization activity. The reduction in bypass efficiency was accompanied by an up to 11-fold increase in the incorporation of complementary nucleotides relative to noncomplementary nucleotides. The fidelity of DNA synthesis, measured by copying a gapped M13 DNA template *in vitro*, was also enhanced as much as 15-fold; the enhancement resulted from a decrease in transitions, which were relatively frequent, and a large decrease in transversions. Our demonstration that an amino acid substitution within the active site enhances the fidelity of DNA synthesis by hPolη, one of the most inaccurate of DNA polymerases, supports the hypothesis that even error-prone DNA polymerases function in base selection.

Accurate DNA replication is required for the maintenance of a species. The accuracy of cellular DNA replication results from the exceptionally high fidelity of DNA synthesis by replicative DNA polymerases and multiple mechanisms for the repair of DNA damage. As a result, the mutation rate in human cells has been estimated to be as low as 1×10^{-10} mutations/nucleotide/cell division (1). Replicational accuracy is maintained despite the large number of lesions caused by both exogenous and endogenous DNA damaging agents. For example, evidence suggests that spontaneous depurination of DNA can result in 10,000 abasic sites per cell per day (2), and an even larger number of lesions are produced as a result of DNA damage by reactive oxygen species (3). Many of the lesions are likely to escape DNA repair mechanisms and therefore are present in DNA templates during copying by replicative DNA polymerases. Bulky lesions can block the progression of DNA replication, forcing the cell to employ additional mechanisms to complete replication in the presence of unrepaired lesions (4, 5).

Recently, a new family of DNA polymerases, referred to as the Y-family, has been identified and categorized as translesion synthesis DNA polymerases. The Y-family of DNA polymerases is highly conserved through evolution. Thus far, four Y-family DNA polymerases have been identified in human cells: DNA polymerase η (hPolη)[1] (6, 7), DNA polymerase κ (hPolκ) (8), DNA polymerase ι (hPolι) (9), and the DNA-dependent dCMP transferase Rev1 (10). In contrast to replicative polymerases, these DNA polymerases are capable of bypassing bulky DNA lesions, have low processivity, and are highly error-prone in copying undamaged DNA (11, 12).

The primary amino acid sequences of Y-family polymerases bear no discernable homology to those of other DNA polymerases. However, four crystal structures of three Y-family DNA polymerases reveal an overall architecture that is similar to that of other DNA polymerases (13–16). The structures resemble a modification of the right hand-shaped enzyme described for other DNA polymerases, with "thumb," "palm," and "finger" domains. A fourth unique domain is referred to as the "little finger" in Dpo4, the "wrist" in Dbh, and the polymerase-associated domain in yeast Polη (13, 15, 16). The structure of the palm domain in the Y-family DNA polymerases is similar to that in the high fidelity DNA polymerases. In contrast, the finger and thumb domains are smaller and differ significantly from those of the high fidelity enzymes (17), resulting in a reduction of the surface area in the active site that interacts with DNA. Interestingly, the O-helix present in high fidelity DNA polymerases is absent in the Y-family; the O-helix region is believed to enhance fidelity by tightening the surface formed between the incoming nucleotide and template DNA (18). The more open active site of the Y-family DNA polymerases can accommodate 2 nucleotides (13, 15), allowing bypass of bulky lesions such as *cis-syn* thymine dimers and resulting in a greater frequency of misincorporation in copying natural DNA templates (19).

The gene for hPolη is mutated, and the protein is inactive, in the cancer-prone genetic disorder xeroderma pigmentosum

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[1] The abbreviations used are: hPolη, human Polη; Polη, DNA polymerase η; XPV, xeroderma pigmentosum variant syndrome.

variant syndrome (XPV) (6, 7). XPV patients are 1000 times more susceptible to sunlight-induced skin cancer than are normal individuals (20). Purified hPolη synthesizes past *cis-syn* thymine-thymine (T-T) dimers *in vitro* with efficiency approaching that observed for undamaged DNA, inserting primarily complementary adenines opposite the lesions (6, 7). The cancer-prone phenotype of XPV patients can be explained by the diminished accuracy of T-T dimer bypass caused by the lack of hPolη. The polymerase domain of hPolη resides in the N-terminal 513 amino acids of the 713 amino acid polypeptide (15, 21). The C-terminal sequence is less conserved but contains regions that are essential for polymerase function *in vivo*, including a nuclear localization signal (22, 23) and protein-protein interaction domains including a proliferating cell nuclear antigen binding site (24) and residues that interact with Polι (25). To gain greater understanding of structure-function relationships in hPolη, we investigated the ability of hPolη Tyr-52 mutants to bypass *cis-syn* T-T dimers, as well as the fidelity of synthesis on an undamaged DNA template.

MATERIALS AND METHODS

Construction and Selection of Active hPolη Tyr-52 → X Mutants—Twenty site-directed reactions were performed with pYEX-hPolη (26) to create all 19 amino acid substitutions for Tyr-52 and the amber mutant, yielding pYEX-hPolη Y52Xs (Y52V and Y52N exhibited low expression in the *in vitro* synthesis system (see below) and were not further characterized). M59 *Saccharomyces cerevisiae rad30rad52* cells were transformed with 19 pYEX-hPolη-Y52*X* plasmids (including Tyr-52 → Stop) and exposed to 50 J/m^2 of UV-C radiation as described (26). Each survival experiment was performed at least three times.

DNA Polymerase Assays—DNA polymerase activity and bypass were measured in primer extension assays. Five nM ^{32}P-5′-end-labeled template-primer (26) was incubated at 30 °C for 15 min (or as indicated) in 10-μl reaction mixtures containing 2 μl of a 1:20 dilution of *in vitro*-synthesized enzyme (26) or 0.1 nM purified enzyme, 100 μM each of the four dNTPs in Polη buffer (40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 60 mM KCl, 10 mM dithiothreitol, 250 μg bovine serum albumin, 2.5% glycerol). Reactions were terminated by the addition of 20 μl of 98% formamide, 10 mM EDTA; 5 μl of each product was analyzed by electrophoresis through 14% denaturing polyacrylamide gels and quantified by phosphorimaging analysis.

Fidelity Assay and Kinetic Analysis—Kinetic analysis of misinsertion was carried out with 50 nM template-primer in extension reactions. Incubation was at 30 °C for 5 min (hPolη) or 30 min (hPolη Y52E) in 10 μl of total volume containing 1 nM purified enzyme, 0–4 μM correct dATP, 0–10 μM incorrect dGTP, 0–1000 μM incorrect dCTP or dTTP. At least duplicate determinations were performed for each polymerase at 10 different dNTP concentrations. Kinetic constants were derived as described (27). The oligomers used in the kinetic analysis were: template, 5′-CCC GGG AAA TTT CCG GAA TTC GAT ATT GCT AGC GGG AAT TCG GCG CG-3′; primer, 5′-[^{32}P]-CGC GCC GAA TTC CCG CTA GCA AT-3′.

Gap-filling Synthesis and Fidelity Measurements—Bacterial strains, reagents, and preparation of substrates were as described (28, 29). Reactions (20 μl) contained 40 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 250 μg/ml bovine serum albumin, 60 mM KCl, 2.5% glycerol, 10 mM $MgCl_2$, 1 mM dNTPs, 150 ng of M13mp2 DNA with a 5-nucleotide gap, 1 mM ATP, 400 units of T4 DNA ligase, and 20 nM hPolη or hPolη Y52E. Reactions were incubated at 37 °C for 1 h and terminated by adding EDTA to 15 mM. DNA products were processed for determination of mutant frequencies as described (30).

Cloning of cDNAs Encoding the hPolη Polymerase Domain—cDNAs corresponding to the polymerase domains (amino acids 1–513) of wild-type hPolη and mutants Y52A, Y52E, Y52F were subcloned into the expression vector pET-15B (Novagen) to yield pET-15B-hPolη Y52*X*. Expression conditions and purification were as described (26). The predicted fusion protein sequence was verified by DNA sequencing.

Molecular Modeling—Coordinates for the recently determined structures of P2 DNA polymerase IV (Dpo4) in a ternary complex with template, primer, and incoming nucleotide (13) (Protein Data Bank code 1JX4) and of yeast DNA polymerase η (15) (Protein Data Bank code 1JIH) were obtained from the Protein Data Bank (31). The molecules were visually superposed using the Cα atoms of the palm domains with the program "O" (32). Yeast pol η was used as a model for hPolη, and the single site mutations were made using the SEQ/EDIT/PROTEIN/MUTATE feature in the program suite Molecular Operating Environment, MOE (Version 2002.03), available from the Chemical Computing Group (Montreal, Canada, www.chemcomp.com). After using O to roughly reorient the side chain of the mutated residue, the coordinates were energy-minimized in MOE. Only atoms within 15 Å of the mutated site Y64E were minimized. Minimization energy parameters from the Koll94 set (33) were used with default weights, except that planar restraints were weighted 100 times more. Hydrogen positions were not calculated, but chiral centers were restrained to their current value. The MOE energy minimization procedure carried out 50 steepest descent, 50 conjugate gradient, and 100 truncated Newton iterations for a rough minimization.

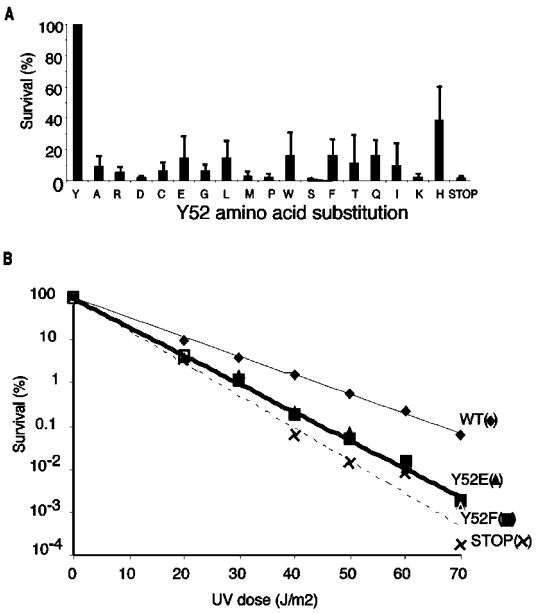

FIG. 1. **Tyr-52 substitutions, resistance to UV-irradiation.** *A*, the effect of hPolη Tyr-52 mutants on UV sensitivity of a *S. cerevisiae rad30rad52* strain that lacks both host Polη (Rad30p) and Rad52p. The percent survival (mean ± S.D.) of transfectants at 50 J/m^2 is shown relative to wild-type enzyme. *B*, survival curves for wild-type (*WT*) hPolη, hPolη Y52E, hPolη Y52F, and a negative control (stop mutant). Results are the average of two independent experiments. D_{37} values (J/m^2), derived from the curves obtained in the two experiments, are: wild type, 9.2, 10.3; Y52E, 5.7, 7.0; Y52F, 6.2, 7.0; stop mutant, 4.8,6.1.

RESULTS

We have previously established a library of 200,000 mutants of human Polη harboring random nucleotide substitutions in codons corresponding to motif II in the finger domain. From this library, we identified mutants that can complement the UV sensitivity of a *S. cerevisiae rad30* (yPolη) *rad52* strain. Complementation is dependent on both catalytic activity and the ability of the mutant to rescue the yeast from UV sensitivity. Sequencing of 200 active mutants established that all 22 amino acid positions that were randomized tolerated multiple substitutions without loss of complementation, except for Tyr-52 and Ala-54 (26). Here we describe the effects of 18 amino acid replacements for Tyr-52 on the function of hPolη *in vivo* and *in vitro*.

Replacement of Tyr-52 Reduces Activity in Vivo—To investigate the role of Tyr-52 in the function of hPolη, we first assessed the essentiality of Tyr-52 for survival after UV irradiation. We used site-directed mutagenesis to construct 18 different plasmids, each containing nucleotide substitutions encoding a different amino acid in place of Tyr-52. The plasmids were transfected into UV-sensitive *S. cerevisiae rad30rad52* cells (34). Survival was measured after exposing transfectants to 50 J/m^2 of UV irradiation. As shown in Fig. 1*A*, expression of wild-type hPolη enhanced UV resistance as much as 100-fold at this dose. All substitutions at Tyr-52 resulted in

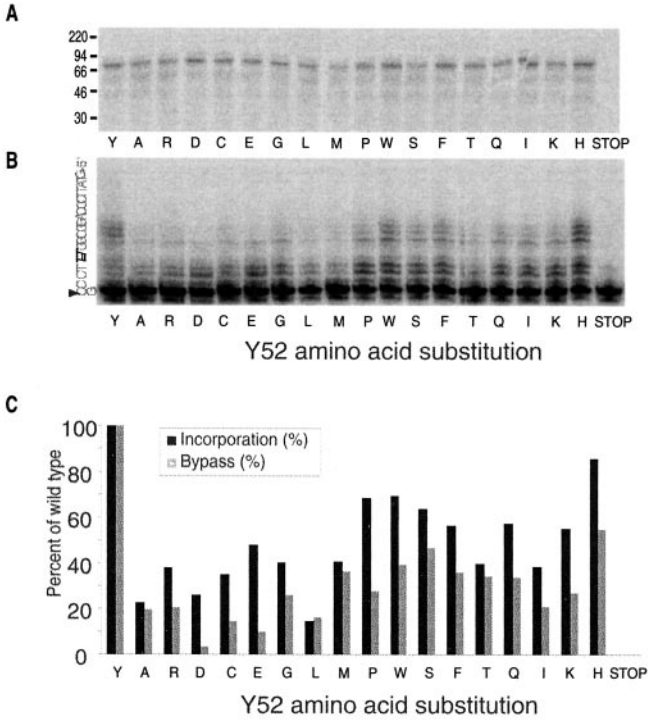

FIG. 2. **Bypass of *T-T* dimers *in vitro*.** *A*, *in vitro* transcription and translation of hPolη Tyr-52 variants in the presence of ^{35}S-labeled methionine. *B*, *in vitro* bypass of a site-specific *T-T* dimer by *in vitro*-synthesized hPolη Tyr-52 variants. The template lesion (indicated by *TT*) is 4 nucleotides downstream of the 3′-primer terminus (indicated by the *right arrow*). C, phosphorimaging analysis of polymerase and bypass activities relative to wild type. DNA polymerase activity was calculated as the sum of the intensities of the extended primers; each of the bands was weighted by a factor equal to the number of nucleotides incorporated. Bypass activity was calculated as the sum of the intensities of all primers extended past the target dimer (*i.e.* 6–14 nucleotides incorporated) divided by the intensity of the primer extended to and past the target (*i.e.* 3–14 nucleotides incorporated). The calculation was normalized to the amount of enzyme in reaction mixtures, as measured by ^{35}S label.

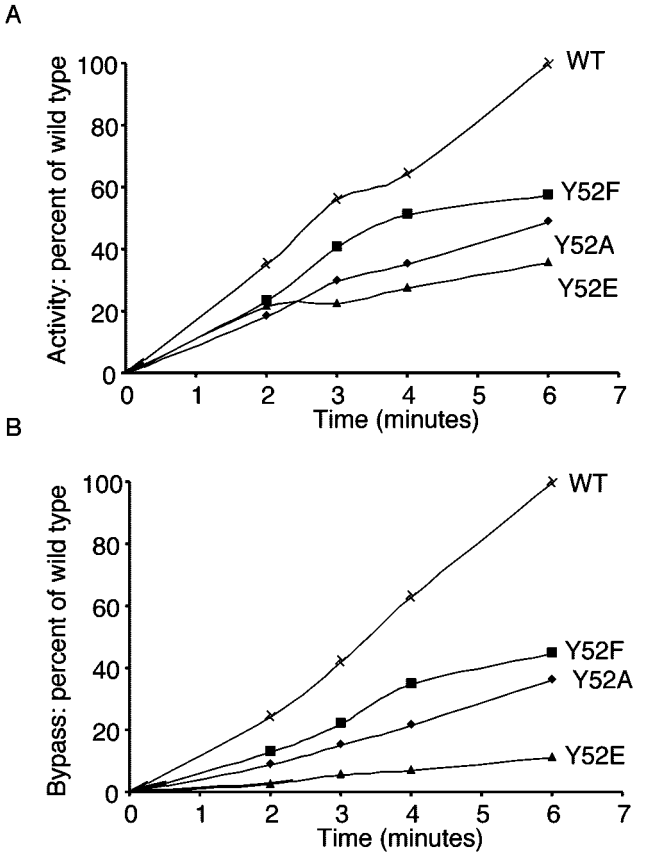

FIG. 3. **Translesion synthesis by purified hPolη wild-type (*WT*) and Tyr-52 mutant proteins on a template containing a T-T dimer.** Bypass of a site-specific T-T dimer was analyzed in assays containing 0.1 nM purified wild-type, Y52A, Y52E, or Y52F polymerase domain. *A*, quantification of polymerase activities by phosphorimaging analysis. DNA polymerase activity was calculated as the sum of the intensities of the 14 bands corresponding to extended primers; each of the bands was weighted by a factor equal to the number of nucleotides incorporated. *B*, quantification of bypass by phosphorimaging analysis. Bypass was expressed as the sum of the intensities of all primers extended past the target dimers (*i.e.* 6–14 nucleotides incorporated) divided by the intensity of the total primer in the reaction. To compare bypass and activity, the data were presented as a percent of wild-type activity or bypass obtained at 6 min.

reduced survival relative to that conferred by wild-type hPolη. Survival curves for the Y52E and Y52F mutants (Fig. 1*B*) revealed greater killing than that observed for the wild-type enzyme at all doses examined. These data provide a direct demonstration of decreased biological activity conferred by substitution at Tyr-52 and substantiate the inability to recover replacements for this amino acid by functional complementation of UV sensitivity (26). The findings thus emphasize the importance of Tyr-52 in the bypass of UV-induced DNA damage *in vivo*.

Activity of Tyr-52 Substitutions in Vitro—To assess the effects of substitutions at Tyr-52 on catalytic activity and lesion bypass, we synthesized wild-type hPolη and 18 mutant proteins in a coupled transcription-translation system (26). Synthesis was carried out by using plasmids encoding the wild-type and mutant proteins as templates for PCR amplification. The amplified hPolη cDNAs, under control of a T7 promoter, were then used as templates for *in vitro* transcription and translation in the presence of ^{35}S-labeled methionine. The wild-type and mutant preparations displayed comparable purity in SDS-polyacrylamide gels, exhibiting a single major band (78 kDa) (Fig. 2*A*). The polymerization and bypass activities of the *in vitro*-synthesized proteins were assayed by using an oligonucleotide template-primer containing a site-specific T-T dimer located 4 nucleotides downstream from the 3′-OH primer terminus (Fig. 2*B*). Polymerase activity varied widely among the mutants, ranging from about 15% of wild type to a level approximately equivalent to wild type (Fig. 2*C*). However, none of the mutants displayed bypass activity equivalent to that of wild-type hPolη (Fig. 2*C*), as was also the case for UV survival (Fig. 1*A*). Bypass activity among the mutants ranged from 10% or less than that of wild type (Y52D and Y52E) up to about 55% of wild type (Y52H).

In Vitro Activity of Purified Tyr-52 Mutant Proteins—To further characterize the bypass efficiency of the hPolη Tyr-52 mutants, we purified several mutant proteins. By using site-directed mutagenesis, we constructed plasmids that efficiently expressed the polymerase domain of wild-type hPolη and the mutants Y52A, Y52F, and Y52E. Substitution with Ala or Phe deletes the aromatic ring structure and hydroxyl group of Tyr, respectively. Glu was chosen because it exhibits greatly reduced bypass and is more similar in size to Tyr than is Asp. The proteins were overexpressed in *Escherichia coli* and purified to greater than 95% homogeneity. Absence of exonuclease activity was confirmed for each preparation in reactions with 8-fold more enzyme than a template-primer with a 3′-G/T mismatch. No shortening of the primer was observed in the absence of dNTPs after incubation for 20 min at 30 °C. The bypass activities of the purified proteins were analyzed in the same primer extension assay described above, with an oligonucleotide tem-

TABLE I
Fidelity of hPolη and hPolη Y52E, incorporation opposite template T

	V_{max}	K_m	V_{max}/K_m	F_{inc}
	nM/min	*mM*		
hPolη				
dATP	4.0 ± 2.6	1.7 ± 0.7	2.35	1.0
dCTP	2.2 ± 1.5	55 ± 29	0.04	1.65×10^{-2}
dGTP	3.6 ± 1.0	3.2 ± 0.1	1.13	4.76×10^{-1}
dTTP	2.1 ± 1.2	38 ± 21	0.06	2.28×10^{-2}
hPolη Y52E				
dATP	1.3 ± 0.5	3.93 ± 1.5	0.33	1.0
dCTP	0.5 ± 0.2	448 ± 144	0.001	3.76×10^{-3}
dGTP	3.5 ± 3.2	32.74 ± 13	0.11	3.33×10^{-1}
dTTP	2.1 ± 1.1	3129 ± 1123	0.001	2.09×10^{-3}

TABLE II
Base substitution specificity for hPolη and for hPolη Y52E while conducting short gap-filing DNA synthesis
The substrate template sequence is 5′-GT*TGA*-3′. Multiple changes occurred in 3 out 67 mutants of the wild-type enzyme and 2 out 66 mutants of hPolη Y52E.

Template nucleotide	Mismatch template-dNTP	Revertant nucleotide	hPolη		hPolη Y52E	
			Number of mutants	Error rate[a] $\times 10^{-3}$	Number of mutants	Error rate[a] $\times 10^{-3}$
T	T-dTTP	A	3	4.9	0	<0.1
	T-dGTP	C	22	36	24	2.6
	T-dCTP	G	1	1.6	2	0.2
G	G-dGTP	C	2	3.3	2	0.2
	G-dATP	T	1	1.6	0	<0.1
A	A-dGTP	C	6	9.8	3	0.3
	A-dCTP	G	22	36	30	3.2
	A-dATP	T	7	11.4	3	0.3

[a] Error rates are calculated as the number of instances a change occurs divided by the total number of mutants sequenced, multiplied by the total mutation frequency, and divided by 0.6 to account for the probability of the transformed cells expressing the error.

plate-primer containing a T-T dimer at the fourth and fifth bases downstream from the primer terminus. For all four polymerases, the accumulation of products extended past the dimer was linear with time (Fig. 3*A*). Wild-type hPolη exhibited highly efficient synthesis past the T-T lesion, and the mutant enzymes maintained the same order of bypass efficiency as observed with the *in vitro*-synthesized enzymes (hPolη > Y52F > Y52A > Y52E) (Fig. 3, *A* and *B*). Activity paralleled bypass efficiency, exceptions being hPolη Y52E and Y52D. hPolη Y52E exhibited a 2.5-fold reduction in polymerase activity as compared with hPolη and a 10-fold reduction in bypass efficiency.

hPolη Y52E Has Higher Fidelity than Wild-type hPolη—Polη is reported to be one of the most error-prone DNA polymerases in copying unaltered DNA templates (30, 35). The disparity between bypass efficiency and polymerase activity exhibited by Y52D and Y52E brought forth the possibility that incorporation of mispaired nucleotides might also be decreased. We examined the fidelity of the Y52E mutant in copying undamaged DNA. In the DNA context we used, the catalytic efficiency (V_{max}/K_m) of the wild-type enzyme for insertion of the non-complementary pyrimidines dCTP and dTTP opposite a template T was 50-fold less than for incorporation of complementary dATP, due primarily to increases in K_m (Table I). Catalytic efficiency for incorporation of the incorrect purine, dGTP, was only slightly less than that for the correct purine, dATP. This high misincorporation frequency is consistent with the average frequency of 10% found for the similar sequence context (ATA) by Matsuda *et al.* (36). hPolη Y52E displayed a significant increase in selectivity for incoming nucleotides as compared with wild-type hPolη. Catalytic efficiency for insertion of correct dATP, as well as for incorrect dGTP, was reduced 7–10-fold relative to values for the wild-type enzyme. The reduction was greater, about 33–82-fold relative to wild type, for insertion of incorrect pyrimidines dCTP and dTTP. Based on relative catalytic efficiencies (F_{inc}, Table I), the Y52E mutant incorporated the non-complementary nucleotides dCTP, dGTP, and dTTP 4.4-, 1.4-, and 10.9-fold less efficiently than wild-type hPolη. The enhanced selectivity of nucleotide insertion exhibited by Y52E resulted mostly from 10–100-fold increases in K_m. These findings suggest that the Y52E substitution confers enhanced discrimination against misinsertion of pyrimidine as compared with purine nucleotides opposite the template T examined.

Differences in Mutational Spectra of Wild-type and hPolη Y52E—To analyze the fidelity for both nucleotide insertion and extension of the misincorporated nucleotide, we measured the ability of both hPolη and hPolη Y52E to fill a 5-nucleotide gap in double-stranded M13mp2 DNA (30). The gap contained a TGA nonsense codon in the *lacZα* gene. Faithful synthesis and ligation results in DNA products that yield colorless M13 plaques on indicator plates containing isopropyl-1-thio-β-D-galactopyranoside. Errors are scored as blue revertant plaques on the basis of α-complementation in the indicator *E. coli* strain. This assay scores for eight of nine possible single base substitutions at the TGA stop codon. The reversion frequencies for the DNA products of this synthesis were 6.5% (1419 of 21,719 plaques) and 0.43% (1000 of 235,950 plaques) for hPolη and hPolη Y52E, respectively. Thus, hPolη Y52E is 15-fold more accurate than the wild type during short gap-filling DNA synthesis. Sequence analyses of 67 (wild type) and 66 (Y52E) individual *lacZ* mutants (64 single mutants, plus 3 and 2 multiple mutants, respectively) recovered from synthesis by each enzyme indicated that both the wild-type and Y52E polymerases catalyzed substitution errors at each of the three phenotypically detectable target nucleotides. However, substitutions did not occur randomly. For both the wild-type (44 of 64 single mutants) and mutant (54 of 64 single mutants) enzymes (Table II), most of the revertants were transitions consistent with the two possible mispairs, T·dGTP and A·dCTP. Transversions can result from six of eight putative mispairs. However, these account for only 20 of 64 revertants for wild type and 10 of 64 revertants for hPolη Y52E. Thus, the enhancement in

fidelity conferred by the Y52E substitution resulted from a 12-fold decrease in the frequency of transitions, which were the most commonly occurring mutations, and a larger, 25-fold decrease in the frequency of transversions, which were less prevalent. As noted, these values reflect the accuracy of both insertion and extension.

DISCUSSION

Multiple mechanisms have evolved to repair the large numbers of DNA lesions produced in human cells by environmental agents and by the generation of reactive molecules produced by normal metabolic processes. Yet it has long been recognized that many lesions escape DNA repair and are present in cells at the time of DNA replication. Thus, it has been difficult to understand how high fidelity replicative DNA polymerases with highly structured catalytic sites can copy past these lesions, particularly altered nucleotides with bulky adducts. The discovery of a new family of DNA polymerases, the Y-family, has provided an answer to this question. The Y-family polymerases have the ability to copy past a variety of DNA lesions that block synthesis by replicative DNA polymerases. Additionally, these enzymes only incorporate one or a few nucleotides with each binding event, lack a proofreading exonuclease, and are error-prone in copying unaltered DNA templates. The presence of multiple Y-family DNA polymerases in human cells raises questions as to how each DNA polymerase copies past different lesions in cells and how these different polymerases are recruited to stalled DNA replication forks (37, 38). One member of this family, Polη, is particularly efficient at polymerizing nucleotides opposite UV-induced *cis-syn* thymine-thymine dimers and other DNA lesions.

Although similar to other DNA polymerases, significant structural modifications appear to distinguish the Y-family from previously characterized polymerases. Unlike the replicative polymerases, the more open and solvent-accessible binding pocket of bypass polymerases studied to date allows for the accommodation of 2 nucleotides or bulky adducts (15). This may account for the reduced fidelity exhibited by these polymerases. In more accurate DNA polymerases, the finger domain plays a crucial role in maintaining fidelity during polymerization. A segment of the finger domain, the O-helix, has numerous contacts with the incoming dNTP that contribute to proper positioning in the active site and to accuracy (39). In the Y-family DNA polymerases, there is no O-helix in the finger domain. Instead these polymerases contain a truncated finger consisting of a loop between two small α-helices.

In this work, we analyzed the role of conserved amino acid Tyr-52 in hPolη in the bypass of T-T dimers and in the fidelity of DNA synthesis. We combined site-directed mutagenesis with our yeast-based complementation system (26) to show that substitution of Tyr-52 with any other amino acid reduces cell survival after UV-induced damage. The mutants harboring replacements for Tyr-52 exhibited from 2- to 20-fold reductions in the ability to bypass T-T dimers. The Y52D and Y52E mutants showed a relatively large reduction in bypass relative to polymerase activity, whereas the Y52H mutant showed the least reduction. One of the mutants, Y52E, exhibited a 9-fold reduction in bypass capability and a 10–15-fold greater fidelity than that of wild-type hPolη.

Tyr-52 is located in the "substrate lid" of the finger domain. Superposition of the crystal structure of yPolη (15) on a ternary complex of Dpo4 containing polymerase, primer, template, and incoming nucleotide (13), and subsequent superposition of the hPolη sequence in this region reveals that the side chain of Tyr-52 faces into the active site cavity and may directly or indirectly affect the interaction with incoming bases (Fig. 4*A*). Due to the truncated thumb and finger domains, the Y-family

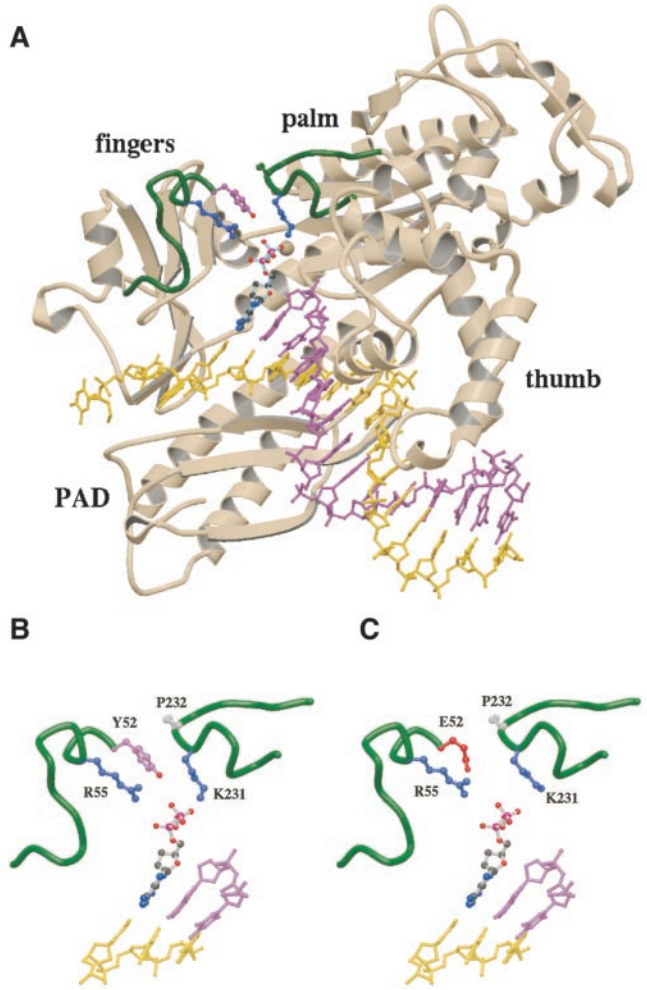

FIG. 4. **Superposition of yPolη structure with primer, template, and incoming nucleotide from Dpo4 structure and model of hPolη Y52E.** *A*, yPolη (*putty color*) backbone, with primer (*lilac*), template (*yellow*), and incoming nucleotide (*atom coloring*) from P2 DNA polymerase IV (Dpo4) structure (13). Side chains of Tyr-64 (52 in human pol η), Arg-67 (55 in human pol η), Pro-280 (232 in human pol η), and Lys-279 (231 in human pol η) are shown in *ball-and-stick* with residue coloring (*lilac*, aromatic; *blue*, Lys/Arg; and *green*, Pro). The orientation of the figure is the same as the close-up view. *PAD*, polymerase-associated domain. *B*, close-up view of the region near Tyr-64 (52 in human pol η) in yPolη. *C*, close-up view of Y52E, yPolη Tyr-64 (52 in human pol η) superposed with mutant Glu-64 (52 in human pol η). The amino acid number in *parentheses* corresponds to hPolη. Positively and negatively charged residues are colored *blue* and *red*, respectively.

DNA polymerases lack the tight fit and high degree of nucleotide discrimination that result from intimate contact between the finger domain, the template, and incoming nucleotides. The region in the finger domain of Polη that interacts with the template substrate nucleotide includes the αD, αE helices and the loop between them. Analogous to yeast Polη Tyr-64, Tyr-52 is likely sandwiched between the highly conserved Arg-55 in the fingers domain and Pro-232 in the palm domain. Although there are many other interactions forming the interface between these two domains, hydrogen bonding between Tyr-52, water, and phosphate of the incoming nucleotide, as well as between Arg-55 and the phosphate of the incoming nucleotide, suggest that maintenance of the relative orientations of these two residues is crucial for reactivity. (Fig. 4*B*) (40). Consistent with this, multiple amino acid substitutions for Tyr-52 result in severe reduction of bypass ability. This reduction could occur by altering the structure of the active site.

Replacement of tyrosine with glutamic acid (Y52E) reduces

bypass activity while increasing the accuracy of nucleotide selection. It should be noted that in our model (Fig. 4*B*), the phenolic side chain of Tyr-52 is distant from the incoming base and is in closer proximity to the β- and γ-phosphates. The large flat side chain of Tyr-52 could help maintain the orientation of Arg-55, which interacts with the phosphate groups of incoming nucleotides, whereas the hydroxyl of Tyr-52 could also hydrogen-bond to phosphate via a solvent molecule. Glutamic acid in the Y52E mutant could both pack less well with the flat Arg side chain, as well as introduce a negative charge that would compete with phosphate for the positively charged Arg side chain. (Fig. 4*B*).

The correct orientation of the incoming dNTP is essential for efficient nucleophilic attack by the 3′-OH group of the primer terminus on the α-phosphate of the incoming dNTP. Alteration in fidelity could be mediated by an overall reorganization of the catalytic site of the polymerase or by a change in the chemical step that affects the fidelity of synthesis. A simple interpretation of the enhancement in specificity is that specific base-pairing interactions become relatively more important if the rate of bond formation becomes rate-limiting as the active site geometry is degraded by mutations. It is likely that introduction of a negative charge, poorer hydrogen bonding, and removal of the well packed planar side chain all contribute to the neighboring arginine being less optimally oriented for good reactivity. The Tyr to His replacement functions because it is a planar substitution with some hydrogen bonding capacity, albeit not as optimal as the conserved Tyr. It is generally assumed that a conformational change in the polymerase is rate-limiting for each nucleotide addition step. Such a change would have to be different for each base-pairing, but recent fluorescent studies of DNA polymerase-β have failed to detect a rate-limiting conformational change (41). Furthermore, Fersht (42) has presented arguments that a rate-limiting conformational change would not enhance specificity without altering the transition state. Thus, the enhancement in fidelity we observe in the Y52E mutant would be in accord with a rate-limiting chemical step that affects the fidelity of DNA synthesis.

The fidelity of hPolη is much lower than that of replicative DNA polymerases (12). In fact, the error rate of this polymerase is similar to that which could be achieved by the differences in free energy between correct and incorrect base pairings (1 error in 10 to 1 error in 100) (43). Thus, it is conceivable that hPolη simply links incoming nucleotides together, rather than functioning in base selection. Our demonstration that an amino acid substitution within the active site enhances the fidelity of DNA synthesis by hPolη, one of the most inaccurate of DNA polymerases, supports the hypothesis that even error-prone DNA polymerases function in base selection.

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