Active Site Mutations in Mammalian DNA Polymerase δ Alter Accuracy and Replication Fork Progression*

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DNA polymerase δ (pol δ) is one of the two main replicative polymerases in eukaryotes; it synthesizes the lagging DNA strand and functions in DNA repair. In previous work, we demonstrated that heterozygous expression of the pol δ L604G variant in mouse results in normal life span and no apparent phenotype, whereas a different substitution at the same position, L604K, is associated with shortened life span and accelerated carcinogenesis. Here, we report in vitro analysis of the homologous mutations at position Leu-606 in human pol δ. Four-subunit human pol δ variants that harbor or lack 3′ → 5′-exonuclytic proofreading activity were purified from Escherichia coli. The pol δ L606G and L606K holoenzymes retain catalytic activity and processivity similar to that of wild type pol δ. pol δ L606G is highly error prone, incorporating single noncomplementary nucleotides at a high frequency during DNA synthesis, whereas pol δ L606K is extremely accurate, with a higher fidelity of single nucleotide incorporation by the active site than that of wild type pol δ. However, pol δ L606K is impaired in the bypass of DNA adducts, and the homologous variant in mouse embryonic fibroblasts results in a decreased rate of replication fork progression in vivo. These results indicate that different substitutions at a single active site residue in a eukaryotic polymerase can either increase or decrease the accuracy of synthesis relative to wild type and suggest that enhanced fidelity of base selection by a polymerase active site can result in impaired lesion bypass and delayed replication fork progression.

Eukaryotic DNA replication is extraordinarily accurate, with an average of less than one error per 109–1010 bases copied (1). The high fidelity of DNA synthesis is principally determined by the properties of the replicative nuclear DNA polymerases, DNA polymerase (pol)2 δ and DNA pol ε (2, 3). These enzymes are highly accurate with respect to nucleotide selection at the polymerase active site, with additional accuracy conferred by an associated 3′ → 5′-exonuclease proofreading domain, which excises noncomplementary nucleotides immediately after incorporation (4). The overall fidelity of DNA synthesis is further enhanced by post-replication repair (5). Accurate DNA replication is central for the avoidance of cancer, a disease that arises in association with the accumulation of mutations in multiple genetic pathways controlling cellular growth and metastasis (6). Given that many mutations are necessary for the development of cancer, yet that normal DNA replication is extremely accurate, it has been postulated that error prone synthesis via the acquisition of a mutator phenotype may be required for carcinogenesis (7, 8). Because pol δ and pol ε are responsible for the majority of DNA syntheses during each replicative cycle, we infer that mutations in the genes encoding these enzymes that render DNA synthesis error prone could constitute a major mechanism for the expression of a mutator phenotype during carcinogenesis. This postulate has been investigated by creating mice expressing mutant variants of pol δ or pol ε that lack 3′ → 5′-proofreading exonuclease activity. In the case of both pol δ (9) and pol ε (10), exonuclease-deficient heterozygous mice have a near normal life span and no apparent phenotype, whereas homozygous animals exhibit a substantially elevated tumor incidence. These studies suggest that the exonuclease domains of the replicative polymerases have an important role in suppressing tumorigenesis. However, these exonucleases likely have functions in the cell other than proof-reading of replication errors, such as maturation of Okazaki fragments, processing of mismatch repair intermediates (11), and removing nucleotide modifications that prevent the initiation of DNA synthesis by DNA polymerases (12). Defects in these processes might also contribute to the observed cancer phenotypes.

Our group recently developed a second model for analyzing the relationship between the fidelity of DNA synthesis by pol δ and carcinogenesis. We have previously demonstrated that substitutions for isoleucine at homologous positions 709 and 614 in Escherichia coli DNA polymerase I (13) and Thermus aquaticus DNA polymerase I (14), respectively, alter the fidelity of DNA synthesis. These studies were extended to Saccharomyces cerevisiae; we created all 19 amino acid substitutions for the homologous position in the catalytic site of S. cerevisiae pol δ (15). This work identified variants that enhance mutagenesis in yeast. We then selected two of these variants, L604G (pol δ5) and L604K (pol δ5), for further study in mice (16). Mice homozygous for either mutation are inviable. Heterozygous pol δ5 mice lack a distinctive phenotype; they have a normal life

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2 The abbreviations used are: pol, polymerase; PCNA, proliferating cell nuclear antigen.

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span and no change in cancer incidence. In contrast, heterozygous pol δ+/K mice exhibit a shortened life span and accelerated tumorigenesis. The overall cancer incidence, as well as the spectrum of tumor types, was similar among pol δ+/+, pol δ+/G, and pol δ+/K mice. However, the rate of tumor progression was greater in the pol δ+/K animals; they died with tumors at an earlier age.

Available mutation data indicate that both pol δG and pol δK result in altered fidelity of DNA replication in vivo. In S. cerevisiae, the frequency of CAN1 resistance in cells expressing pol δG is 17-fold elevated relative to wild type, whereas pol δK yielded a somewhat lower 13-fold elevation (15). In mouse embryonic fibroblasts, fluctuation analysis revealed that the mutation rate at the HpRt locus is elevated 5-fold relative to wild type in cells expressing pol δG, whereas cells expressing pol δK exhibit a 4-fold elevation (16). Given that the pol δG mutation does not result in a cancer phenotype in heterozygous mice, whereas the pol δK mutation is associated with accelerated tumorigenesis, it is apparent that the overall magnitude of elevation in the mutation rate is not sufficient to explain the phenotype in mice. However, the spectrum of mutations synthesized by the variants differs. In S. cerevisiae, the identified replication errors in pol δG cells consist predominantly of point mutations (95% point mutations; 5% deletions). In contrast, cells expressing pol δK exhibit an increased frequency of deletions (65% point mutations; 35% deletions) (15). Likewise, in mouse embryonic fibroblasts, chromosomal spreads revealed that although cells from both pol δ+/G and pol δ+/K mice exhibit an elevated frequency of spontaneous chromosomal aberrations relative to wild type, the highest levels of aberrations are seen in pol δ+/K cells, which are elevated 2–3-fold relative to pol δ+/G cells (16). These differing patterns of mutation in vivo are likely relevant to the disparate phenotypes of the pol δG and pol δK mice, but their etiology is not clear as the biochemical properties of the pol δG and pol δK polymerases are unknown.

The study of mutated mammalian pol δ has previously been hampered by the lack of an efficient purification system. A recently described method (17) now allows for sufficient amounts of human pol δ to be obtained to allow for detailed biochemical analysis. Thus, to study the biochemical consequences of mutation in the active site of mammalian pol δ and by extension gain insight into the disparate phenotypes of the pol δ L604G and pol δ L604K mouse models, we have purified the homologous variants at position Leu-606 of human pol δ. We report that human pol δG and pol δK maintain polymerase and exonuclease activity, replicate DNA with comparable processivity, and are similar in the extent to which their activity is stimulated by PCNA. However, pol δG and pol δK differ dramatically in base replication accuracy. pol δG synthesizes DNA harboring an extremely high frequency of point mutations, which is an unexpected finding as mice expressing a single copy of the homologous mutation do not have a discernible phenotype. In contrast, the pol δG variant, which is associated with a heterozygous cancer phenotype in mice, results in highly accurate synthesis in vitro; surprisingly, the accuracy of base selection by the active site of pol δG is improved relative to that of wild type pol δ. However, pol δK is defective in bypassing sites of DNA damage in vitro, and expression of the homologous variant in mouse embryonic fibroblasts results in stalled replication in vivo. We conclude that substitution at a single residue in the active site of human pol δ can either increase or decrease the accuracy of base selection and that enhanced base selection accuracy is associated with impaired bypass of bulky DNA adducts. Extension of our results to the pol δα and pol δK mouse models suggests that reduced DNA synthesis by a major replicative polymerase can result in genetic instability and accelerated carcinogenesis.

EXPERIMENTAL PROCEDURES

Expression and purification of human pol δ and the polymerase and exonuclease assays were performed as described previously (18). pol δ catalytic mutants were constructed by site-directed mutagenesis of the pET303-hpolD1 plasmid with the Stratagene QuikChange kit. Human PCNA was a kind gift of Prof. Ulrich Hübcher (19).

**Processivity Assay**—Reactions (30 µl) contained 600 fmol of plus-strand m13 DNA annealed to 5'-32P end-labeled primer 5'-GCTGTTGGAGGGCGATCG-3', 40 mM Tris-HCl (pH 7.5), 50 mM KC1, 2.5 mM MgCl2, 0.1% Triton X-100, 10% glycerol, 6 µg of BSA, 1 mM DTT, 250 µM of each dNTP, and 30 fmol of exonuclease-proficient DNA polymerase. Reactions were initiated by addition of polymerase and incubated for 15 min at 37°C. Length of extension products was constant at 5, 10, and 15 min, indicating that single-hit conditions were satisfied (data not shown). Reactions were quenched by adding an equal volume of 95% formamide, 20 mM EDTA, and products were separated on a 14% denaturing sequencing gel.

**Stimulation by PCNA**—Stimulation of pol δ activity by PCNA was performed essentially as described previously (20). In brief, reactions (10 µl) contained 400 fmol of poly(dA)-poly(dT)30, 0.1 pmol of exonuclease-proficient DNA polymerase, 0.3 pmol of human PCNA, 40 mM Tris-HCl (pH 7.9), 8 mM MgAc2, 2 µg of BSA, 1 mM DTT, and 25 µM [α-32P]dTTP. Reactions were quenched by addition of EDTA to 25 mM final concentration. Products were then bound to a Microdyne B membrane (Nalge Nunc International, Rochester, NY) and quantified by PhosphorImage analysis.

**Single Nucleotide Kinetics**—Reactions utilized 5'-32P end-labeled primer 5'-CAT GAA CTA CAA GGA C-3' annealed to the template 5'-GCA TTC AGT GGT CCT TGT AGT TCA TG-3'. Reactions (10 µl) contained 40 mM Tris-HCl (pH 7.5), 50 mM KC1, 5 mM MgCl2, 0.1% Triton X-100, 10% glycerol, 2 µg of BSA, 1 mM DTT, 10 mM primer-template, and 20 nM of the indicated exonuclease-deficient DNA polymerase. Reactions were initiated by nucleotide addition, proceeded 1–10 min at 37°C as needed to satisfy single completed hit conditions with less than 20% total extension of available primer (21), and were quenched with an equal volume of 95% formamide, 20 mM EDTA. Reaction products were heated to 95°C for 5 min, separated on a 14% polyacrylamide-urea gel, and quantified by PhosphorImage analysis. Reaction velocity was plotted as a function of nucleotide concentration and fit to the Michaelis-Menten equation with the KaleidaGraph software package.

**Forward Mutation Assay**—Reactions (25 µl) contained 40 mM Tris-HCl (pH 7.5), 50 mM KC1, 2.5 mM MgCl2, 0.1% Triton
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X-100, 10% glyceral, 5 μg of BSA, 1 mM DTT, 250 μM of each dNTP, 1.5 mM M13mp2 gapped DNA substrate, and 300 nM polymerase. Reactions were initiated by incubation at 37 °C for 1 h and terminated by addition of EDTA to 10 mM. Reaction products were introduced into E. coli; mutant plaques were scored, and error rates were calculated as described previously (18).

Lesion Bypass Assay—The substrate was 5′-[32P] end-labeled primer 5′-CGC GCC GAA TTC CCG CTG CTA GCA ATA TTC T-3′ annealed to the template 5′-TTG GCN GCA GAA TAT TGC TAG CGG GAA TTC GGC GCG-3′, where N indicates the modified nucleotide as indicated in the figure legend. Reactions (10 μl) contained 40 mM Tris-Cl (pH 7.5), 50 mM KCl, 2.5 mM MgCl2, 0.1% Triton X-100, 10% glycerol, 5 mM DTT, 250 μM of each dNTP, 5 μM of primer-template, and 60 μM of exonuclease-proficient DNA polymerase. Reactions were incubated for 5 min at 37 °C, quenched with an equal volume of 95% formamide, 20 mM EDTA, heated to 95 °C for 5 min, and separated on a 14% polyacrylamide-urea gel.

Molecular DNA Combing—Iododeoxyuridine, chlorodeoxyuridine, and SDS were obtained from Sigma. Proteinase K was from Roche Diagnostics. Secondary antibodies for molecular combing were from Fisher. Cells were incubated with 50 μM iododeoxyuridine for 20 min followed by 50 μM chlorodeoxyuridine for an additional 20 min in the culture medium. Thymidine chase was then carried out at 1 mM for 1 h. Cells were embedded in agarose plugs and treated with 1 mg/ml proteinase K, 1% SDS in 0.5 M EDTA (pH 8.0) for 48 h. DNA was combed on silanized coverslips as described previously (22). Primary antibodies were described previously (23) except the anti-single-stranded DNA antibody was used at 1:100 (Chemicon Inc). Secondary antibodies were used at 1:25 (anti-rat Alexa-594), 1:150 (anti-mouse Alexa-488), 1:100 (Chemicon Inc). Secondary antibodies were used at 1:50 (anti-mouse Alexa-488), 1:150 (anti-mouse Alexa-350), and 1:25 (anti-rat Alexa-594). Signals were captured and measured as described previously (23). Fork speed was calculated by dividing the median track size by the labeling time. Differences in fork speed between individual cell lines were examined using the Mann-Whitney test, which is a nonparametric equivalent of the t test and provides a measure of identity of two independent populations.

RESULTS

Purification of pol δ Active Site Mutants—Four-subunit human pol δ variants were purified from E. coli by the method of Fazlieva et al. (17). The purification scheme involves transformation of E. coli with two plasmids: one plasmid expresses the 125-kDa catalytic subunit, and the other plasmid expresses the p66, p50, and p12 accessory subunits. A polyhistidine tag on the 125-kDa subunit allows the complex to be isolated on a 125-kDa column in vivo

FIGURE 1. Polymerase and exonuclease activity of pol δ variants. Polymerase activity was assayed by incorporation of radioactive nucleotides into activated calf thymus DNA, and exonuclease activity was determined on a radioactively labeled single-stranded poly(dT) homopolymer. Assays were performed in triplicate and normalized to wild type pol δ standard deviations are indicated.
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δ activity by enhancing its ability to bind DNA (27). We measured the effect of PCNA on the activity of each pol δ variant when copying a primed DNA substrate (poly(dA)-oligo(dT)) (supplemental Fig. S3). On this template, in the absence of PCNA, wild type pol δ and pol δ<sup>K</sup> had similar activity, whereas pol δ<sup>G</sup> had somewhat increased activity, which could indicate an increased capability for pol δ<sup>G</sup> to replicate through areas of secondary structure that are known to occur in homopolymeric templates (28). In the presence of PCNA, 4-subunit wild type pol δ, pol δ<sup>G</sup>, and pol δ<sup>K</sup> were all stimulated to comparable overall levels of synthesis. This result is consistent with a report indicating that the interaction between pol δ and PCNA is mediated predominantly by the p50 accessory subunit of pol δ and not the p125 catalytic subunit (29), which suggests that mutations in p125 would not be expected to alter the pol δ-PCNA interaction.

Single Nucleotide Kinetics—Nucleotide selection by the pol δ<sup>G</sup> and pol δ<sup>K</sup> mutants relative to wild type pol δ was analyzed by measuring the kinetics of incorporation of a single correct or incorrect nucleotide onto the 3′-primer terminus of a DNA substrate (21). To focus on base selection at the active site, exonuclease-deficient versions of the enzymes were utilized in the single nucleotide incorporation experiments. The rate of insertion of a single dCTP residue opposite template dG by pol δ is shown in Fig. 2. Wild type pol δ, pol δ<sup>G</sup>, and pol δ<sup>K</sup> catalyzed this insertion event with similar efficiency (Fig. 2A), consistent with the comparable activities of the three variants on activated calf thymus DNA. The rate of nucleotide mis-insertion was investigated by measuring incorporation of dTTP opposite template dG (Fig. 2B). Wild type pol δ catalyzed mis-insertion of dTTP at low but detectable levels, with a maximum reaction velocity of 0.80 fmol/min. pol δ<sup>G</sup> catalyzed the mis-insertion event at a significantly higher rate of 6.5 fmol/min, indicating reduced accuracy of nucleotide selection at the polymerase active site. Although the <i>V</i><sub>max</sub> for dG:dTTP insertion differed between wild type pol δ and pol δ<sup>G</sup>, the <i>K</i><sub>m</sub> values for this event were identical within experimental error. Under the steady-state conditions used in the assay, the <i>K</i><sub>m</sub> approaches the nucleotide binding constant and can be considered to represent the residence time of nucleotides in the enzyme-DNA-dNTP complex (30). Thus, the comparable <i>K</i><sub>m</sub> values we observed suggest that wild type pol δ and pol δ<sup>G</sup> have comparable residence times for mismatched dNTPs at the dNTP-binding site. This observation is consistent with our previously published molecular modeling studies concerning the L604G and L604K variants of mouse pol δ (16), which suggested that the substitutions do not greatly alter the active site geometry and thus might not be expected to alter dNTP association/dissociation. The mechanism by which the motif A substitutions affect the fidelity of DNA synthesis may therefore involve an alteration in the conformational change that is thought to occur during nucleotide incorporation at the polymerase active site (31, 32).

pol δ<sup>K</sup> did not catalyze detectable nucleotide mis-insertion, even with nucleotide concentrations as high as 4 mM. This lack of detectable mis-insertion by pol δ<sup>K</sup>, in contrast to that observed with wild type pol δ and pol δ<sup>G</sup>, suggests that the lysine substitution reduces incorporation of noncomplementary bases at the active site. Incorporation was insufficient to determine whether the reduced single nucleotide mis-insertion by pol δ<sup>K</sup> is concordant with a change in <i>K</i><sub>m</sub> or <i>V</i><sub>max</sub>. Fidelity of DNA Synthesis—To quantify the fidelity of each pol δ variant for all possible base substitution errors, we utilized the M13-lacZ gapped DNA forward mutation assay (33). This assay utilizes double-stranded M13 DNA containing a 407-nucleotide single-stranded gap in the sequence encoding the lacZα-complementation fragment. The single-stranded region is copied <i>in vitro</i> by the polymerase. Complete gap filling is confirmed by agarose gel electrophoresis, and the products are transformed into α-complementation E. coli cells followed by plating on media containing X-gal and isopropyl 1-thio-β-D-galactopyranoside. Correct synthesis by the polymerase results in the production of β-galactosidase and the formation of dark blue plaques, whereas polymerase errors that inactivate the lacZα gene result in light blue or colorless plaques. The ratio of light blue or colorless plaques to dark blue plaques reflects the overall fidelity of the polymerase; sequencing of mutant plaques allows determination of the mutational signature of the polymerase within multiple sequence contexts.
All of the pol δ mutants copied the entire gapped segment of the plasmid (data not shown). As reported previously (18), wild type pol δ replicates DNA with high accuracy, resulting in mutant plaques at a frequency of 1.1 × 10⁻³ ± 0.6 × 10⁻³ (average of duplicate experiments ± S.D.). This frequency is only slightly greater than the background lacZ mutant frequency of 0.7 × 10⁻³. The error frequency of the pol δ⁹ variant is 50-fold greater, with a mutant plaque frequency of 53 × 10⁻³ ± 11 × 10⁻³ (Fig. 3). Inactivation of proofreading further increases the mutant frequency by less than 2-fold to 98 × 10⁻³ ± 3 × 10⁻³. This observation implies that the mutator phenotype induced by the L606G mutation is primarily due to an alteration in base selection at the active site rather than altered proofreading or impaired polymerase-exonuclease domain switching (34), although these mechanisms may contribute to the inaccuracy of the L606G mutant as well. The elevated mutation frequency in the L606G,D402A double mutant additionally demonstrates that proofreading only partially compensates for the error prone mutant active site.

In sharp contrast, the pol δ⁸ variant yielded a lacZ mutant frequency of 1.7 × 10⁻³ ± 0.2 × 10⁻³, which is near the background of the assay and not substantially elevated relative to wild type pol δ. Moreover, when proofreading is inactivated in the L606G,D402A double mutant, the lacZ mutant frequency of 2.3 × 10⁻³ ± 0.6 × 10⁻³ is 4-fold lower than that seen with inactivation of proofreading alone (9.2 × 10⁻³ ± 0.8 × 10⁻³). The difference between pol δ L606K,D402A and pol δ D402A is significant (p < 1 × 10⁻⁴, Fisher’s exact test). This observation suggests that the L606K substitution enhances the accuracy of DNA synthesis, which is consistent with the results seen in the single nucleotide incorporation assay.

The types of mutations introduced by the exonuclease-proficient pol δ variants were determined by sequencing the copied segment of gapped DNA in the lacZ mutant plaques (for detailed mutation data, please see supplemental Table S1). pol δ⁸ introduced a large number of mutations throughout the lacZ target sequence (Fig. 4). We observed an increase in mispair frequency relative to wild type for every possible single-base substitution error other than dCMP mis-incorporation opposite template C. This indicates that the L606G mutation causes an overall reduction in the fidelity of DNA synthesis and that the lowered fidelity is expressed in multiple sequence contexts. In contrast, synthesis by pol δ⁸ results in a low frequency of errors throughout the lacZ target (Fig. 4). Both base substitution and single-nucleotide framenashes are seen at a rate similar to that of wild type pol δ and approximately equal to the background of the assay; this implies that most or all mutations observed for pol δ⁸ are likely due to pre-existing mutations in the DNA substrate rather than being caused by errors during gap-filling synthesis. Although the frequency of deletions with pol δ⁸ was somewhat elevated relative to wild type in this in vitro assay, this difference was not significant (p = 0.13, Fisher’s exact test).

**Lesion Bypass**—The alterations in nucleotide selectivity seen in the Leu-606 mutant enzymes might affect the ability of the enzymes to copy DNA templates containing altered nucleotides. We measured the ability of the exonuclease-proficient pol δ variants to replicate a template containing a site-specific lesion at the +3 position (Fig. 5A). On a control template, slight differences in activity were seen among the wild type, L606G, and L606K variants, although all exhibited sufficient activity to extend beyond the +3 position. In the presence of a template lesion, the wild type pol δ stalls but is able to incorporate nucleotides opposite 8-oxoguanine, 1,N⁶-ethenoadenine, O⁶-methylguanine, O⁶-methylthymine, and a synthetic abasic site (tetrahydrofuran), with a partial ability to continue synthesis past the lesions. pol δ⁸ differs from wild type by exhibiting an increased ability to both incorporate opposite and continue extension beyond all the site-specific modifications. This observation is consistent with the reduced accuracy of nucleotide selection by pol δ⁸, which is presumably due in part to relaxed geometry at the active site of the mutant polymerase during the nucleotide addition step. pol δ⁸, on the other hand, was reduced in lesion bypass activity relative to wild type. Reactions with pol δ⁸ resulted in nearly complete termination of DNA synthesis at the position prior to the site-specific modification for each lesion analyzed. Quantification of the lesion bypass efficiency of each pol δ variant is shown in Fig. 5B; bypass efficiency of each variant was normalized to the extent of synthesis seen on the undamaged template. We observed that synthesis by pol δ⁸ was impaired most prominently when encountering 8-oxoguanine, which is a common lesion due to reactive oxygen species in vivo and is readily bypassed by most DNA polymerases (31); diminished synthesis by pol δ⁸ relative to wild type was also observed for the alkylated lesions O⁶-methylguanine and O⁶-methylthymine. Minimal extension was observed beyond ethenoadenine and abasic sites for all of the polymerases tested.

**Replication Fork Progression**—We hypothesized that a diminished ability of the pol δ polymerase to copy past altered nucleotides would be manifested in impaired progression of the replication fork in vivo due to stalled synthesis at endogenous sites of DNA damage. We probed this postulated mechanism by measuring replication fork progression on a genome-wide scale by molecular combing (22, 23). We sequentially labeled exponentially dividing mouse embryonic fibroblasts
with the nucleotide analogs iododeoxyuridine and chlorodeoxyuridine, isolated total DNA, and stretched the DNA molecules on silanized coverslips. Neo-replicated tracks were revealed with fluorescent antibodies specific for the nucleotide analogs. Distributions of fork velocity values, as presented in Fig. 6, were obtained from track size analysis as described under “Experimental Procedures.” The fork velocity in cells expressing pol $\delta^{G}$ was unchanged in distribution relative to a lineage-matched control ($p = 0.190$ and $p = 0.306$, nonparametric Mann-Whitney test). In contrast, the distribution of fork velocity in cells expressing pol $\delta^{K}$ exhibited a significant shift toward reduced fork speeds relative to control cells ($p = 5 \times 10^{-3}$ and $p = 3.8 \times 10^{-3}$). These results imply that DNA synthesis in cells expressing pol $\delta^{K}$ is impaired, consistent with stalling of replication secondary to impaired bypass of endogenous lesions in DNA by the mutant pol $\delta$.

**DISCUSSION**

The expression of the four-subunit complex of human pol $\delta$ in *E. coli* has facilitated biochemical studies of mutant mammalian pol $\delta$ (17, 18) and has allowed for analysis of the consequences of substitutions in the active site of a mammalian replicative DNA polymerase. Our studies indicate that both the L606G and L606K substitutions in human pol $\delta$ result in retained polymerase and exonuclease activity, unchanged processivity, and intact interaction with PCNA. However, the two variants differ greatly in their base selection accuracy; human pol $\delta^{G}$ is extremely error prone, incorporating single noncomplementary nucleotides at a 50-fold elevated rate relative to wild type pol $\delta$. In contrast, pol $\delta^{K}$ is highly accurate, with base selection by the active site that is more faithful than that of wild type pol $\delta$. To our knowledge, this study represents the first demonstration of a substitution in a mammalian polymerase that results in improved accuracy of

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**FIGURE 4. Mutation spectrum of pol $\delta$ mutants.** The + strand coding sequence of the single-stranded portion of lacZa is shown, with point mutations indicated above the line and deletions ($\Delta$) or insertions below. Top panel, pol $\delta$ L606G; bottom panel, pol $\delta$ L606K.
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In vitro bypass of DNA lesions is responsible for the observed defect in replication fidelity of pol δ. The enhanced base selection accuracy by DNA polymerases has not been optimized during evolution for maximal fidelity (35, 36). The enhanced base selection fidelity of pol δ L606K is associated with an apparent defect in bypass of DNA lesions in vitro, as well as impaired progression of the replication fork in mouse cells in vivo. Small differences in lesion bypass efficiency could have a major impact on DNA replication in vivo when one considers the large number of lesions that are likely to be encountered by a replicative polymerase, and we hypothesize that stalling of pol δ L606K at DNA lesions is responsible for the observed defect in replication fork progression.

The catalytic subunit of human pol δ is 94% conserved with mouse pol δ, and the sequence of motif A is identical between the two organisms. Thus, our results with mutated human pol δ in vitro may be relevant to understanding the phenotype seen in mice expressing the homologous variants in vivo. Heterozygous expression of the highly error prone pol δG variant in heterozygous mice, pol δL604G, and pol δL604K mouse embryonic fibroblasts. The wild type cell line used in each experiment was derived from a cross of heterozygous animals. Box, 25–75 percentile range. Whiskers, minimum and maximum values. Med, median fork speed (kb) ± median absolute deviation; n, number of measurements; p, p value determined with nonparametric Mann-Whitney test.

The impaired replication fork progression that was seen in cells expressing pol δK, which is highly accurate in vitro, may be due to an increased role during nuclear DNA replication of the error prone bypass polymerases such as pol η and pol κ (37), secondary to the impaired lesion bypass capability of pol δK. In contrast to the differing phenotypes seen upon expression of the two variants in heterozygous mice, pol δG and pol δK are both embryonic lethal upon homozygous expression. The lethality of these polymerases in homozygous mice, despite the near-wild type catalytic activity of the purified enzymes in vitro, suggests the accumulation of a lethal burden of mutations that is not compatible with mammalian development (10).

The elevated frequency of point mutations in cells expressing pol δK, which is highly accurate in vitro, may be due to an increased role during nuclear DNA replication of the error prone bypass polymerases such as pol η and pol κ (37), secondary to the impaired lesion bypass capability of pol δK.
of the DNA damage that occurs in vivo is likely to occur to free nucleotides prior to their incorporation into DNA (38), and thus this interpretation is also consistent with the overall hypothesis that synthesis by pol δK is impaired in vivo due to a defect in the ability of the mutant polymerase to process DNA damage.

Notably, in our previous work with pol δ+/G and pol δ+/K mouse embryonic fibroblasts (16), we did not detect a difference between the variants in activation of the DNA damage response as assayed by serine 139 phosphorylation of histone H2AX. However, activation of the checkpoint response is likely to occur only in response to near-lethal levels of DNA damage, and thus the partial defect in lesion bypass synthesis we observed by pol δK in vitro may be sufficient to result in accumulation of deleterious DNA rearrangements over multiple cellular generations, without causing an absolute block to synthesis and thus without widespread activation of the checkpoint response. We hypothesize that cells expressing pol δK will exhibit a disproportionate increase in the induction of replication fork stalling and genomic rearrangements when lower levels of DNA damage are induced in cells. However, it is also possible that factors that were not studied in this work may also contribute to the observed in vivo defect in DNA synthesis by pol δK, such as sequence-specific stalling at endogenous replication barriers such as structured non-B DNA and common fragile sites (39, 40).

The ability of the replicative DNA polymerases to copy past limited amounts of damaged DNA without interruption is likely a requirement for efficient progression of the replication fork. In addition to DNA damage in cells induced by environmental processes, DNA is modified by endogenous reactive molecules generated by normal cellular metabolism, and in particular by reactive oxygen species (41). It has been estimated that 1% of oxygen metabolism results in reactive oxygen species (42) and that reactive oxygen species induce ~20,000 damaged nucleotides in each cell per day (43). Although cells possess specialized repair processes that are induced in the presence of significant levels of damage, these processes are unlikely to be sufficient to remove the thousands of damaged bases that are likely present in most cells at the time of genome replication. In addition, failed DNA repair can result in intermediates that are more detrimental than the initial lesion (44, 45). During DNA replication, pol δ is likely to encounter many blocking lesions; although eukaryotic cells do possess lesion bypass polymerases that are able to take over for the replicative polymerases and replicate beyond sites of damage that have not yet undergone repair, the catalytic efficiency of the major replicative polymerases is much greater than that of the bypass polymerases, and as a result small alterations in bypass by pol δ may have profound effects in cells. Thus, mutations in pol δ (such as L606G) that enhance the ability to bypass lesions might be better tolerated than mutations in pol δ (such as L606K) that reduce bypass.

Overall, pol δG and pol δK exhibit a similar burden of point mutations in vivo, yet a cancer phenotype is seen only in the case of pol δK, which exhibits impaired lesion bypass in vitro as well as stalled replication and large genomic rearrangements in vivo. These observations may be relevant to understanding the relative contributions of point mutations versus genomic rearrangements in spontaneous carcinogenesis. DNA sequencing of human cancers has revealed large numbers of mutations in each tumor analyzed. Presently, every tumor reported has at least 50 clonal mutations that alter the coding sequence of genes (46); fully sequenced DNA from human tumor has been reported to contain ~30,000 clonal somatic mutations (47). Moreover, there are no mutations that are invariably present in any type of tumor, and there is little evidence for a series of mutations that are diagnostic of any particular type of tumor (46). Nearly all of the reported mutations in tumors are single-base substitutions, predominantly transitions (48, 49). An increase in transition mutations can be considered as a hallmark of mis-incorporation by replicative DNA polymerases α, δ, and ε (1, 50) but can also be the result of DNA damage by reactive oxygen species. Many mutations identified in tumors are likely to be passenger mutations that have arisen during the last round of clonal selection. Evidently cells are able to tolerate large numbers of single-base substitutions, even those that result in nonsynonymous amino acid changes.

In contrast to single-base substitutions, rearrangements can result in frameshift mutations and large deletions that excise multiple contiguous genes and regulatory regions of DNA. Large deletions can cause inactivation of the corresponding genes on the homologous chromosome resulting in loss of heterozygosity, which is a characteristic of many tumors (51). Thus, per mutagenic event, deletions would be predicted to have a more profound effect on cellular phenotype than would single-base substitutions. The relative importance of DNA deletions compared with point mutations is supported by investigations on the fidelity of mitochondrial DNA replication. Substantial circumstantial evidence links mitochondrial DNA mutations to the aging process (52). A 500-fold increase in single-base substitutions in mouse mitochondria results in a far greater burden of point mutations than is seen in vivo, yet with no apparent phenotype (53). The high tolerance to elevated point mutations in the mitochondrial genome is particularly striking when one considers its small size, with only 16 kb of sequence, and its extremely high density of coding sequence. The nuclear genome, with its substantial prevalence of noncoding sequence, would thus be expected to have an even higher tolerance to random point mutations. In contrast, a modest increase in random mitochondrial DNA deletions is associated with a dramatic accelerated aging phenotype (54), suggesting that mitochondrial DNA deletion events may be a driver of mammalian aging. We propose that nuclear DNA rearrangements may play a similarly central role in cancer progression.

Large genomic rearrangements are a known hallmark of cancer. However, smaller insertions and deletions that are present in individual cells within tumors are difficult to detect by current DNA sequencing methods. Even though massive DNA sequencing has revolutionized the way we think about many disease processes, its application in studying cancer may be limited by the heterogeneity of cells within a tumor. Accurate quantitation of these alterations would require long read lengths or the sequencing of single molecules. Moreover, most rearrangements that arise during tumorigenesis would likely either be lethal or would be negatively selected during tumor progression.
progression. The rate at which rearrangements occur in tumors is likely to be much higher than sequencing would predict, and random insertions and deletions that are not detected by current technology may represent an important driving force in tumor initiation and progression.

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