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Human Pol ϵ -Dependent Replication Errors and the Influence of Mismatch Repair on Their Correction

Anderson Ayuk Agbor^{a,b}, A. Yasemin Göksenin^a, Kimberly G. LeCompte^a, Samuel H. Hans^a, and Zachary F. Pursell^{a,b,*}

^aDepartment of Biochemistry and Molecular Biology, Tulane University School of Medicine, 1430 Tulane Ave., New Orleans, LA 70112, USA

^bTulane Cancer Center, Tulane University School of Medicine, 1430 Tulane Ave., New Orleans, LA 70112, USA

Abstract

Mutations in human DNA Polymerase (Pol) ϵ , one of three eukaryotic Pols required for DNA replication, have recently been found associated with an ultramutator phenotype in tumors from somatic colorectal and endometrial cancers and in a familial colorectal cancer. Possibly, Pol ϵ mutations reduce the accuracy of DNA synthesis, thereby increasing the mutational burden and contributing to tumor development. To test this possibility *in vivo*, we characterized an active site mutant allele of human Pol ϵ that exhibits a strong mutator phenotype *in vitro* when the proofreading exonuclease activity of the enzyme is inactive. This mutant has a strong bias towards mispairs opposite template pyrimidine bases, particularly T•dTTP mispairs. Expression of mutant Pol ϵ in human cells lacking functional mismatch repair caused an increase in mutation rate primarily due to T•dTTP mispairs. Functional mismatch repair eliminated the increased mutagenesis. The results indicate that the mutant Pol ϵ causes replication errors *in vivo*, and is at least partially dominant over the endogenous, wild type Pol ϵ . Since tumors from familial and somatic colorectal patients arise with Pol ϵ mutations in a single allele, are microsatellite stable and have a large increase in base pair substitutions, our data are consistent with a Pol ϵ mutation requiring additional factors to promote tumor development.

Keywords

DNA Polymerase; DNA replication; Mutagenesis; Mismatch repair

*To whom correspondence should be addressed: Tel: 1-504-988-1974; Fax: 1-504-988-2739; zpursell@tulane.edu.

SUPPLEMENTARY DATA

Supplementary Data are available: Supplementary Table 1.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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1. INTRODUCTION

DNA Polymerase (Pol) ϵ is one of three Pols required to carry out eukaryotic DNA replication [1, 2]. In addition to its involvement in leading-strand DNA synthesis, it has been implicated in the repair of damaged DNA and maintenance of epigenetic inheritance [3–10]. Another role has been suggested by immunolocalization studies in human cells, which showed Pol ϵ to be involved in DNA synthesis specifically in late S phase, possibly through replication of heterochromatin [11]. This observation is particularly intriguing in light of recent results demonstrating that one role of Pol ϵ during *S. pombe* replication is to recruit factors required for silencing pericentromeric heterochromatin [6, 10].

A direct role for Pol ϵ in protecting against tumor development was first demonstrated by studies in mice, where homozygous inactivation of proofreading in Pol ϵ (Pol $\epsilon^{\text{exo-}/\text{exo-}}$) caused increased mutagenesis and cancer mortality [12–14]. The most abundant tumors in the homozygous Pol $\epsilon^{\text{exo-}/\text{exo-}}$ mice were gastrointestinal tumors, similar to what is observed in many mice with mutations in mismatch repair genes [15]. While mutations in mismatch repair genes are known to predispose to colorectal cancer, a subset of patients with either large adenomas or early-onset colorectal cancer contains no known mutations in mismatch repair genes [16]. A recent study that carried out whole genome sequencing of tumors from patients with colorectal cancer identified somatic mutations in *POLE*, the catalytic subunit of Pol ϵ [17]. Whole genome sequencing from members of a family predisposed to colorectal cancer, but lacking any defect in mismatch repair pathway genes, also revealed mutations in *POLE* [16]. In each case the tumors are associated with an ultramutator phenotype, but are microsatellite stable (MSS).

Pol ϵ is a member of the B family of DNA polymerases, which share three highly conserved motifs (Motifs A, B and C) that encode the residues responsible for catalyzing the DNA polymerization reaction [18]. Pol ϵ also contains a 3'→5' exonuclease proofreading activity encoded by three motifs (ExoI, ExoII and ExoIII), which are conserved only in the subset of proofreading-proficient Family B Pols. These conserved Exo and Pol motifs all cooperate to ensure the high fidelity of replication seen with Family B Pols [19]. Motifs A and C together contain the three aspartate residues that coordinate the metal ions required for catalysis. Motif A also contributes an invariant tyrosine, Y631 in human Pol ϵ , that is critical in each of the yeast B family Pols for normal cellular growth [20] and contributes to sugar discrimination by steric interference with the extra 3'-OH present on ribonucleotides [21, 22]. The crystal structures of several B family Pols have been solved to date, including Pol δ from yeast and the Pols from RB69 and ϕ 29 bacteriophages [23–25]. In each structure the conserved tyrosine stacks against the sugar of the incoming dNTP and, with several motif B residues on the opposite side, helps form the nascent base pair binding pocket.

While mutation of the conserved tyrosine results in severe growth defects, changing the adjacent residue in a variety of B family Pols from different organisms generally results in alleles with reduced replication fidelity through mechanisms that are incompletely understood [26–33]. While this adjacent residue is conserved as a leucine in almost all B family Pols, Pol ϵ remains an exception, where it is conserved as a methionine in each eukaryotic organism studied to date. Mutator alleles made by changing this adjacent residue

have proven particularly useful for probing *in vivo* polymerase function due in large part to two properties. The first is that substituting the leucine/methionine has minimal impacts on overall DNA synthesis rates [26]. The second is that the overall reduction in replication fidelity is accompanied by changes in error specificity that result in a unique pattern of *in vivo* mutations [9, 34].

Mutations affecting replicative Pol fidelity have also proven useful in revealing a role of the enzyme in genome instability and cancer in mammals. Although the *in vitro* error specificities of the Pol $\epsilon^{\text{exo-}}$ enzyme are similar to those of the Pol $\delta^{\text{exo-}}$ enzyme [35, 36], when introduced into mice the Pol $\epsilon^{\text{exo-}/\text{exo-}}$ and Pol $\delta^{\text{exo-}/\text{exo-}}$ mutations lead to strikingly different tumor spectra, suggesting that the roles played by each Pol may have specific differences with respect to tumorigenesis. Mutation of the conserved Motif A leucine (L604) in mouse Pol δ has differing effects on replication fidelity and tumorigenesis depending on the nature of the substitution [31]. The L604K-Pol δ mutant makes deletion errors at a high rate, but retains high selectivity against base-base mismatches, while the L604G-Pol δ mutant is a strong mutator for base pair substitutions [37]. In mice, the L604K-Pol δ mutant has a shorter lifespan and increased chromosomal abnormalities relative to the L604G-Pol δ mutant [31]. Studies of Pol ϵ function in human cells have been hampered by a lack of mutant alleles available to probe its function. This report describes the construction and properties of such a mutant.

We have constructed a proofreading-deficient, active site mutant of human Pol ϵ (M630G-Pol $\epsilon^{\text{exo-}}$), and shown that the enzyme is a strong mutator *in vitro*, with a preference for mispairs with template pyrimidines. In particular, the M630G-Pol $\epsilon^{\text{exo-}}$ enzyme has a strong bias for T•dTTP and C•dTTP mispairs relative to their respective complementary A•dATP and G•dATP mispairs. The exonuclease activity of the M630G-Pol $\epsilon^{\text{exo+}}$ enzyme is able to proofread essentially all of the increased mispairs. In the absence of mismatch repair, the M630G-Pol $\epsilon^{\text{exo-}}$ allele is also a mutator *in vivo*, even in the presence of endogenous amounts of the wild type Pol $\epsilon^{\text{exo+}}$. The mutations observed in cells expressing M630G-Pol $\epsilon^{\text{exo-}}$ are dominated by A•T to T•A transversions, characteristic of T•dTTP errors. Mismatch repair completely suppresses the observed mutator effect, suggesting that increased Pol ϵ replication errors alone are insufficient to explain their contribution to tumor development.

2. MATERIAL AND METHODS

2.1. Materials

HyClone MEM/EBSS was from Thermo Scientific (Waltham, MA, USA). Trypsin-EDTA and geneticin G-418 sulfate were purchased from Life Technologies (Carlsbad, CA, USA). Mouse anti-FLAG (M2) antibodies were purchased from Sigma (St. Louis, MO, USA). Mouse anti-DNA polymerase ϵ (3C5.1) was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). Immuno-Blot PVDF membrane was purchased from BIO-RAD (Hercules, CA, USA). Fetal bovine albumin was purchased from Atlanta Biologicals (Atlanta, GA, USA).

2.2. Expression and Purification of the Catalytic Fragment of Human M630G-Pol ϵ

An expression vector encoding residues 1–1189 of either the exonuclease-proficient or –deficient catalytic subunit of human Pol ϵ was used in site-directed mutagenesis reactions to change amino acid residue 630 from methionine to glycine. Human Pol ϵ was prepared as described [35]. Briefly, the human Pol ϵ was coexpressed in autoinduction medium [38] with pRK603, which allows coexpression of TEV protease, at 25°C until the culture was saturated. Peak fractions from the HisTrap column were pooled, dialyzed into 50 mM HEPES, pH 7.5, 1 mM DTT, 5% glycerol and bound to SP sepharose. Bound protein was eluted with a 0 to 1 M NaCl gradient. Peak fractions were pooled, dialyzed into 50 mM Tris, pH 7.5, 1 mM DTT, 5% glycerol, 100 mM NaCl and bound to Q Sepharose. Bound protein was eluted with a 100 mM to 1 M NaCl gradient. Peak fractions were pooled, concentrated and passed through a pre-equilibrated Superdex200 size exclusion column. Fractions containing the purified 140 kDa protein were pooled, dialyzed into 50 mM Tris, pH 8.0, 1 mM DTT, 5% glycerol and aliquots were frozen and stored at –80°C.

2.3. In Vitro Primer Extension and Excision Assays

An 18-mer DNA oligo, 5'-CCTCTTCGCTATTACGCC-3', was radiolabeled with ^{32}P at its 5'-end by incubating with T4 polynucleotide kinase (Invitrogen) and γ - ^{32}P -labeled ATP (Perkin Elmer) for 30 min at 37°C. Unincorporated ^{32}P was separated by passage over an IllustraMicroSpin G-25 column (GE Healthcare). Primers used in polymerization assays were annealed to a complementary 45-mer DNA oligonucleotide with the sequence 5'-TTGCAGCACATCCCCCTTCGCCAGCTGGCGTAATAGCGAAGAGG-3' at a final concentration of 2 μM . Reaction mixtures (30 μl) to measure extension contained 50 mM Tris, pH 7.4, 8 mM MgCl_2 , 1 mM DTT, 10% glycerol, 25 μM each dNTP, and 100 nM DNA primer-template. Reaction mixtures to measure excision were identical except dNTPs were withheld. Reactions were started with the addition of 1 nM enzyme and carried out at 37°C.

2.4. In vitro lacZ Mutant Frequency Determination

The *in vitro* lacZ forward mutation assay was performed essentially as described previously [39]. Briefly, double-stranded M13mp2 DNA containing a 407-nt ssDNA gap was used as a substrate in reactions containing 0.15 nM DNA, 50 mM Tris-Cl, pH 7.4, 8 mM MgCl_2 , 2 mM DTT, 100 $\mu\text{g/ml}$ BSA, 10% glycerol, 250 μM dNTPs and 1.5 nM Pol ϵ -M630G at 37°C. DNA was checked for complete gap-filling by 0.8% agarose gel electrophoresis at 60 V for 16 hr. Completely filled product was then transfected into *E. coli* cells, which were used to determine the frequency of light blue and colorless plaques that occurred as a result of mutations arising during DNA synthesis. In this assay, accurate DNA synthesis yields dark blue plaques. LacZ mutant frequencies were calculated from combining at least two independent experiments. DNA from mutant plaques was subsequently purified and the *lacZ* gene was sequenced. Error rates were calculated as described [39].

2.5. In Vivo Cell Culture and Transfection Experiments

The DNA encoding the complete catalytic subunit of human Pol ϵ (residues 1–2286) was amplified by PCR and cloned into pIRES2-EGFP by using baculovirus containing the open

reading frame of *POLE* [described in [40]] as a template. A FLAG tag was added to the N-terminus via PCR.

The Mlh1⁻, MMR-deficient HCT-116 human colorectal cancer cell line (gift from Dr. Prescott Deininger) was grown and maintained at 37°C and 5% CO₂ in MEM/EBSS supplemented with 10% fetal bovine serum. For transfection, HCT116 cells cultured at 70% confluence were transfected with either the DNA encoding the full length pol ε Exo⁻ M630G mutant, cloned into pIRES2-EGFP vector or the pIRES2-EGFP vector alone using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 48 hours post-transfection, cells were trypsinized and expression of the recombinant protein was monitored by both western blot analysis and by fluorescence using fluorescence activated cell-sorting (FACS) analysis using a BD LSR11 FACS Analyzer (BD Biosciences).

2.6. Western Blot analysis

Transfected cells were harvested by trypsinizing and cell scraping. Cells were rinsed in 1X PBS, resuspended in buffer 1 (10 mM HEPES, 10 mM KCl, 1.5 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail) pH 7.5 and homogenized by dounce homogenization. Triton X-100 was added to a final concentration of 1%, mixed well and incubated on ice for 20 min. Lysed cells were pelleted at 14,000 rpm for 10 min and supernatant was collected. Cell pellet was rinsed once by resuspending in buffer 1, followed by centrifugation at 14,000 rpm for 10 min. To isolate nuclear fractions the resulting pellet was resuspended in high salt buffer 2 (20 mM HEPES, 420 mM NaCl, 0.2 mM EDTA, 25 % glycerol, 0.5 mM DTT, 0.5 mM PMSF) to which protease inhibitor cocktail (Roche) was added, homogenized and incubated on ice for 10 min. The homogenate was centrifuged at 14,000 rpm for 10 min and supernatant containing soluble proteins was collected and subjected to a 10% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes and stained with anti-FLAG antibodies and antibodies against DNA polymerase ε (3C5.1).

2.7. Generation of Human Cell Lines Stably Expressing Mutant Pol ε

HCT116 cells transfected with either the M630G-Pol ε^{exo-} mutant or pIRES2-EGFP vector were sorted based on their fluorescence using a BD FACS Aria instrument. Sorted cells were cultured in media supplemented with 500 µg of geneticin for two weeks for stable transfectants. Single individual clones were picked, expanded and maintained in media containing 400 µg of geneticin.

2.8. In Vivo Mutation Rate and Mutant Frequency Measurements

For each cell line analyzed, cells were grown to confluence in two 6-well plates. Cells from one well were harvested and counted to estimate cell number in the remaining wells. For mutation rate measurement, 500 cells from each of the remaining eleven wells were seeded per dish in 3 × 100 mm dishes in media lacking 6-thioguanine (6-TG). These were used to measure plating efficiency. At the same time, 5 × 10⁵ cells from each of the remaining eleven wells were plated in 5 × 100 mm dishes in media containing 6-TG to select for cells that acquired a mutation in the *HPRT1* gene. After 7 days, colonies on the plating efficiency wells were stained with crystal violet and counted. After 12–14 days, the 6-TG resistant colonies were also stained with crystal violet and counted. Mutation rates and 95%

confidence intervals (95% CI) were calculated using the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method [41].

For mutant frequency measurement, cells at 70–80% confluence were transfected with 3 µg plasmid DNA. After 24 hours, cells were trypsinized and counted. 500 cells per clone were seeded in duplicate in 6-well plates in media lacking 6-thioguanine (6-TG) and allowed to grow for 5–7 days to determine plating efficiency. The remaining wells were seeded with 5×10^4 cells in media containing 6-TG and allowed to grow for 12–14 days. After the indicated time, colonies were stained with crystal violet and counted. Mutant frequency was calculated by the following equation: $(\# \text{ 6-TG resistant colonies}) / [(\text{6-TG seeded cells}) \times (\text{plating efficiency scored colonies}) / (\text{plating efficiency cells seeded})]$. Colonies were defined as 50 cells.

2.9. Sequencing HPRT1 Mutations In Vivo

Total RNA was isolated using the Qiagen RNeasy kit (Qiagen) according to the manufacturer's protocol. RT-PCR was performed with SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol using 1 µg of RNA as a template. Primer-specific cDNA was amplified for 32 cycles at an annealing temperature of 60°C using the following HPRT1 primers: 123(fwd) CTTCTCCTCCTGAGCAGTC and 1041 (rev) GCCCAAAGGGAAGTATGATC. From the *HPRT1* sequencing of 6-TG resistant colonies, one clone was found to have exon 2 completely deleted. Exon deletions in HPRT1 have been shown to be caused by splice site mutations [42]. We therefore amplified exon 2 and its flanking region from genomic DNA prepared from the appropriate clone using the following primers: Forward: TTGTTTTCTTACATAATTCATTATCATACC; Reverse: TTACTTTGTTCTGGTCCCTACAGAG.

3. RESULTS

3.1. DNA synthesis and 3'-5' exonuclease activities of a human Pol ε active site mutant

In order to better understand the roles of Pol ε in replication and genome stability in human cells *in vivo*, we characterized for the first time an active site mutant allele in human Pol ε. The effects of this M630G active site mutation on human Pol ε function, *in vitro* DNA synthesis and 3'-5' exonuclease activities were first compared. We used our previously described N-terminal 140 kDa construct [35, 43] that contains each of the six conserved motifs necessary for 3'-5' exonuclease and DNA synthesis activities (Fig. 1A). The equivalent truncated construct of yeast Pol ε was shown to have highly similar replication fidelity to the holoenzyme and is therefore a useful substitute for *in vitro* replication fidelity assays [28]. Site-directed mutagenesis was used to make the M630G mutant in both the exonuclease-proficient (Pol ε^{exo+}) and – deficient (Pol ε^{exo-}) Pol ε expression constructs and each was purified to homogeneity (Fig. 1B). The ability of each enzyme to extend from a radiolabeled primer in the presence of dNTPs was first compared (Fig. 1C). Both the exonuclease-proficient (M630G-Pol ε^{exo+}) and – deficient (M630G-Pol ε^{exo-}) mutant alleles of Pol ε were able to extend from a duplex primer-template substrate with 110% of the activity of the matched wild type (M630) Exo⁺ and Exo⁻ enzymes. When the ability of the mutant enzyme to excise DNA was compared, the M630G-Pol ε^{exo+} enzyme had similar

3'-5' exonuclease activity to the wild type M630-Pol $\epsilon^{\text{exo}+}$ enzyme (Fig. 1D). The wild type (M630-Pol $\epsilon^{\text{exo}-}$) and mutant (M630G-Pol $\epsilon^{\text{exo}-}$) constructs with exonuclease activity inactivated by amino acid substitution (D275A/D277A) showed no excision products.

3.2. In vitro replication fidelity of a Pol ϵ active site mutant

We next measured the replication fidelity of M630G-Pol ϵ using a *lacZ* forward mutation assay to determine error rates for frameshifts and all twelve possible base-base mispairs in a large number of sequence contexts [39]. The M630G substitution in the absence of proofreading (M630G-Pol $\epsilon^{\text{exo}-}$) caused a 14-fold increase in *lacZ* mutant frequency over the wild type, Pol $\epsilon^{\text{exo}-}$ (Table 1, compare 660 to 46×10^{-4}), indicating that M630 strongly influences the intrinsic selectivity of human Pol ϵ . When the proofreading exonuclease activity of the M630G-Pol ϵ mutant was active however (M630G-Pol $\epsilon^{\text{exo}+}$), the mutant frequency was only 1.8-fold higher than the background of the assay (Table 1, compare 14×10^{-4} to 6.5×10^{-4}), indicating that most replication errors made by the M630G-Pol ϵ mutant are efficiently proofread. This is consistent with the robust observed exonuclease activity (Fig. 1D).

In order to examine the error specificity of both the proofreading-proficient and -deficient M630G-Pol ϵ constructs, we sequenced the *lacZ* gene from isolated individual plaques and calculated error rates for each type of error. Sequencing revealed that the M630G substitution strongly increased base pair substitutions, causing an 18-fold increase in the base pair substitution error rate (Fig. 2A) over that of the exonuclease-deficient, wild type Pol ϵ [35]. M630G-Pol $\epsilon^{\text{exo}-}$ also increased the error rates for frameshifts, though to a lesser degree (6.4-fold), with the increase divided equally between single-base insertions and deletions (Fig. 2A). Measuring error rates for the individual mispairs showed an increase over the wild type Pol $\epsilon^{\text{exo}-}$ for every mismatch, though the relative increase was highly variable, ranging from 3.0-fold for G→C and G→T transversions to 90-fold for C→A transversions (Fig. 2B). The single highest absolute error rate was for T→A transversions (130×10^{-5}). M630G-Pol $\epsilon^{\text{exo}-}$ caused a 29-fold increase in error rate for T→A transversions over the wild type Pol $\epsilon^{\text{exo}-}$. T→A transversions are one of the errors least frequently made by Pol δ , the other primary replication Pol [36], marking it as a potentially highly distinguishing error *in vivo*. Three of the four highest error rates observed, T→A, C→A and G→A (Fig. 2B), each involve a mispair with an incoming dTTP, suggesting a common mechanism of fidelity reduction.

We analyzed the position for each sequenced base pair substitution and frameshift error (Fig. 2C). T→A transversions, the errors with the highest error rate, were evenly distributed over 7 of the 16 detectable sites, with no one site having more than 22% of the total T→A transversions. Caution should be used in interpreting these results given the relatively small number of events. In general, however, replication errors made by M630G-Pol $\epsilon^{\text{exo}-}$ were distributed throughout the template, indicative of a general increase in mutagenesis that is less dependent on the specific sequence context.

3.3. Mutagenesis due to a Pol ϵ active site mutant expressed in human cells

Since M630G-Pol $\epsilon^{\text{exo-}}$ was a 100-fold stronger mutator *in vitro* than the endogenous wild type, M630-Pol $\epsilon^{\text{exo+}}$ (Table 1, compare mutant frequencies of 660 vs. 6.5×10^{-4}), and had a unique error signature, we asked whether this mutant allele was sufficient to confer a mutator phenotype *in vivo*. To do this, we cloned the full-length human Pol ϵ catalytic subunit containing an N-terminal FLAG tag (FLAG-p261) into pIRES2-EGFP, a human cell expression vector. Pol ϵ is a holoenzyme comprised of four subunits, the largest of which is encoded by the *POLE* gene, is 261 kDa and contains the catalytic activities [40, 44]. This vector drives co-expression of the target gene and EGFP as separate proteins translated from a single, bicistronic message. This allows monitoring of EGFP fluorescence in live cells to indicate FLAG-p261 expression. Additionally, cells can be sorted based on EGFP fluorescence and subsequently monitored for FLAG-p261 expression by Western blot against either the catalytic subunit or the FLAG tag.

We first asked whether the full-length FLAG-p261 was expressed in human cells transfected with the construct. Western blots of lysates from cells transfected with either pIRES2-EGFP or pIRES2-EGFP containing the FLAG-p261 construct showed an α -FLAG antigen specific to FLAG-p261 (Fig. 3A, left). Probing these cell lysates with antibodies against the Pol ϵ catalytic subunit showed that overall levels of the catalytic subunit slightly more than doubled, suggesting roughly equal abundance of the endogenous, wild type Pol ϵ and the recombinant, mutant Pol ϵ (Fig. 3A, right). We then asked if the reduced fidelity of M630G-Pol $\epsilon^{\text{exo-}}$ could contribute to mutagenesis *in vivo*. To do this, vector alone or vector encoding several FLAG-tagged Pol ϵ constructs were transfected into human cells. Mutant frequencies for the transfected cells were measured using resistance to 6-thioguanine (6-TG), which is acquired through loss-of-function mutations at the *HPRT1* locus (Fig. 3B). M630G-Pol $\epsilon^{\text{exo-}}$ transfection caused a 7-fold increase in mutant frequency over cells transfected with the empty vector. The strength of the *in vitro* mutator effect, the observed levels of recombinant Pol ϵ protein expression and relative increase in mutagenesis *in vivo* seen with the M630G-Pol $\epsilon^{\text{exo-}}$ mutant are similar to those seen with a Pol α active site mutant in the same human cell line, further validating the utility of this model system [45]. As a control, we observed no effect on mutant frequency when the wild type, Pol $\epsilon^{\text{exo+}}$ was transfected. For comparison, expression of the M630-Pol $\epsilon^{\text{exo-}}$ construct, which is proofreading-deficient but wild type at the polymerase active site, caused a 4-fold increase in mutant frequency. These results indicate that proofreading-deficient Pol ϵ led to increased mutagenesis in human cells, even in the presence of the endogenous, exonuclease-proficient enzyme. The exonuclease-deficient active site mutant, M630G-Pol $\epsilon^{\text{exo-}}$, caused an even larger increase in mutagenesis *in vivo*, consistent with the enhanced mutagenesis seen with this mutant *in vitro*.

HCT-116 cells lack Mlh1 and are deficient in mismatch repair, thus any errors made during replication remain uncorrected in these cells [46, 47]. To test whether the increased mutagenesis in cells expressing mutant Pol ϵ was due to errors made during replication, we measured mutant frequency in HCT-116 cells complemented with Mlh1 cDNA [48]. In contrast, expression of Mlh1 reduced mutant frequency by 3-fold and additional expression of M630G-Pol $\epsilon^{\text{exo-}}$ had no effect (Fig. 3B). These results indicate that the Pol ϵ allele

mutator effect was due to replication errors that are normally subject to correction by the mismatch repair system.

One limitation of mutant frequency measurements is that they do not measure the rates of mutagenesis per cell division. In order to measure this, we used fluctuation analysis to measure mutation rates in cell lines stably transfected with M630G-Pol $\epsilon^{\text{exo-}}$. Prior to transfection, cells were grown for successive generations in HAT medium to remove any cells with pre-existing *HPRT1* mutations. To generate these lines, transfected cells were first sorted and collected based on fluorescence since both the empty and Pol ϵ -containing vector express GFP. Sorted cells were then plated and grown in the presence of geneticin for 21 days to select for independent clones. Clones were then probed by Western blot for FLAG, Pol ϵ p261, actin and GFP. We identified multiple stably transfected clones that expressed FLAG-tagged M630G-Pol $\epsilon^{\text{exo-}}$ (Fig. 4). We then measured mutation rate at the *HPRT1* locus using resistance to 6-TG. Fluctuation analysis and the Ma-Sandri-Sarkar Maximum Likelihood Estimator were used to calculate mutation rates [49]. Those cell lines that expressed the mutant Pol ϵ exhibited an increase in mutation rate over cell lines that stably integrated the empty vector (Table 2). That this mutator effect was observed in cells that retain their normal complement of endogenous, exonuclease-proficient Pol ϵ suggests that the M630G-Pol $\epsilon^{\text{exo-}}$ is partially dominant over the wild type enzyme.

In order to determine whether the increase in observed mutation rates was due to mutations made by the M630G-Pol $\epsilon^{\text{exo-}}$, we sequenced the *HPRT1* gene from a collection of independent 6-TG^R colonies expressing either the mutant or the wild type Pol ϵ (Supplemental Table 1). Mutations in the wild type cells were roughly evenly divided between frameshifts and base pair substitutions, with the base pair substitutions dominated by G•C to A•T transitions (Table 3). Frameshifts were divided between the deletion and insertion at a single run of six consecutive guanine bases and a deletion in a run of four consecutive adenines. This error spectrum closely resembles previously published mutation spectra from the same cell line used in these studies [42, 50]. Sequencing the *HPRT1* gene from cells expressing M630G-Pol $\epsilon^{\text{exo-}}$ revealed a dramatic increase in the number of A•T to T•A transversions (Table 3). While none of these errors were observed in the wild type error spectrum, they made up almost half of the sequenced mutations when the mutator Pol ϵ was expressed. A•T to T•A transversions can arise during replication *in vivo* due to A•dATP mispairs on one strand, or T•dTTP mispairs on the opposite strand. T•dTTP errors were the strongest observed replication error made by Exo⁻-M630G-Pol ϵ *in vitro* (Fig. 2B). Taken together, these data suggest that M630G-Pol $\epsilon^{\text{exo-}}$ expression caused an increase in the mutation rate by causing a large increase in T•dTTP errors, likely during replication.

4. DISCUSSION

While much studied in budding yeast, the consequences of reduced DNA synthesis fidelity of the replication DNA polymerase ϵ is not well understood in human cells. We studied this using an active site mutant allele of human pol ϵ , M630G, that is a strong mutator *in vitro* with a unique error signature that includes a strong preference for T•dTTP mispairs. Proofreading is able to remove essentially all mispairs introduced by the enzyme. However, when proofreading is inactivated, the mutant Pol ϵ allele is a mutator in human cells *in vivo*,

even in the presence of the endogenous, exonuclease-proficient wild type enzyme. This mutator effect is due to increased T•dTTP errors made during replication.

The M630G-Pol $\epsilon^{\text{exo-}}$ enzyme shows a unique preference for mispairs opposite template pyrimidines. Five of the six largest error rates occur with a template pyrimidine, regardless of the nature of the incoming dNTP (Fig. 2B). This feature is shared between Pol ϵ mutant alleles in which the Motif A methionine is substituted with a glycine, as both the human M630G-Pol ϵ (this study) and the orthologous yeast M644G-Pol ϵ [9] exhibit this template pyrimidine bias. This feature is also restricted to active site mutant Pol ϵ alleles as wild type Pol α , which lacks intrinsic exonuclease activity, and exonuclease-deficient Pol δ and RB69 Pols all lack this bias [26, 32, 36]. In addition, the M→G Pol ϵ mutant alleles are alone among B family Pols in their strong preference for T→A transversions, which result from dTTP mispaired opposite a template T. Because of the specificity for mutant Pol ϵ alleles, this strong preference to make and extend dTTP•T mispairs is likely due to specific interactions between the DNA, nucleotide and Pol ϵ active site residues. Solving the crystal structure of this enzyme will greatly assist efforts to understand this unique pattern of replication errors.

Two characteristics of the M630G-Pol $\epsilon^{\text{exo-}}$ make it a potentially powerful tool to study the mechanisms of replication fidelity that underlie mutational acquisition during tumorigenesis. First is that the strong enrichment in A•T to T•A transversions is a unique error signature. A•T to T•A transversions are uncommon errors in a wide variety of *in vivo* studies. Studies that used reporter genes to examine mutations caused by replication errors in cell lines derived from tumors showed a paucity of A•T to T•A transversions; including less than 2% of the total overall spontaneous mutations in HCT-116 cells, the same cell line used in these studies [42, 50, 51]. A number of whole-genome and whole-exome sequencing studies from individual tumors of several different types reveal that A•T to T•A transversions are also among the least common mutation [17, 52, 53]. Increases in this error observed in cells expressing the mutant Pol ϵ could then be easily detected over the noise of additional mutational processes. Importantly, in addition to the unique enrichment in A•T to T•A transversions, the error rates for all base pair substitutions are elevated by the M630G-Pol ϵ mutant, but only when proofreading is inactivated. The second characteristic is that the mutator phenotype of the M630G-Pol $\epsilon^{\text{exo-}}$ allele is its partial dominance over the endogenous, wild type Pol $\epsilon^{\text{exo+}}$ enzyme. This suggests that simple overexpression of the mutant allele will be sufficient to observe mutagenesis in cells without modulating expression of the existing Pol ϵ . This property resembles what is now seen in colorectal and endometrial cancer, where a single mutant Pol ϵ allele is associated with an ultramutator phenotype [17].

An unexplained difference between the Pol $\epsilon^{\text{exo-}}$ mouse model and human tumor data is that the ultramutated human tumors arise when a single allele of Pol ϵ is mutated, while mice require a second mutation, either in the remaining Pol ϵ allele or in the Mlh1 MMR gene, to develop tumors [12]. The increased mutant frequency in a genomic transgene seen in heterozygous Pol $\epsilon^{\text{wt/exo-}}$ mice is insufficient by itself to drive tumorigenesis. This discrepancy suggests that other factors in addition to the loss of proofreading in one Pol ϵ allele may contribute to the observed human tumor development. The human M630G-Pol

$\epsilon^{\text{exo-}}$ human Pol ϵ allele is a stronger mutator than the M630-Pol $\epsilon^{\text{exo-}}$ for each of the twelve base-base mispairs, yet addition of a single allele of this powerful mutator is also insufficient to drive a mutator phenotype in human cells with functional MMR. Our results support the idea that Pol ϵ mutations likely contribute to the ultramutator phenotype in human tumors through multiple mechanisms that each increase mutagenesis, including an overall increase in DNA synthesis errors coupled to a decrease in their detection by the mismatch repair system.

One possibility is that if MMR is partially impaired, the presence of the mutant Pol ϵ alleles would lead to an increase in uncorrected base substitution errors. The human *POLE* mutant tumors are MSS [16, 17, 54], indicating that the mismatch repair system is operating on repetitive microsatellite sequences in these tumors. Interestingly however, all but two of the TCGA *POLE* mutant tumors also had one or more non-synonymous changes in MMR genes, including many in *MSH6*, raising the possibility that the mutant Pol ϵ -dependent increase in base pair substitution replication errors may be exacerbated by a specific rather than general MMR impairment in these tumors. While also microsatellite stable, MMR genes from tumors found in the familial *POLE* mutant patients were not sequenced.

Alternative explanations of mutant Pol ϵ contributions to tumor development are also possible. One alternative is based on observations in yeast that MMR correction of different Pol ϵ replication errors varies depending on both the nature of the mismatch and its genomic location [55] and observations in human cells that MMR is influenced by histone modification status [56]. The Pol ϵ replication errors critical for tumor development would then be predicted to be the subset of errors that are only inefficiently corrected by MMR due to their identity and/or genomic context. Interestingly, mutations that alter the Pol ϵ holoenzyme subunit composition in yeast cause increased mutagenesis that is MMR-independent and involves increased use of Pol ζ [57–60]. When both Pol ϵ proofreading alleles are inactivated, all Pol ϵ replication errors might then saturate the MMR system more generally, causing mutations more broadly throughout the genome. The levels of mutagenesis and subsequent tumor development would then be sensitive to the dosage of the mutant Pol ϵ , consistent with the differences seen in non-tumorigenic Pol $\epsilon^{\text{wt/exo-}}$ and pro-tumorigenic Pol $\epsilon^{\text{exo-/exo-}}$ mice [12]. An additional alternative mechanism is through interactions between the mutant Pol ϵ and some DNA lesion(s) not present in the model systems, possibly via oxidative stress induced by chronic inflammation [61]. Each of these proposed mechanisms can be tested using the M630G-Pol $\epsilon^{\text{exo-}}$ allele we describe and will help shed light on how replication defects contribute to cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- An active site mutant of human Pol ϵ is a strong mutator for base pair substitutions.
- Proofreading corrects almost all replication errors made by this mutant.
- The proofreading-deficient mutant drives mutagenesis in MMR-deficient human cells.
- MMR fully able to correct proofreading-deficient Pol ϵ replication errors.
- Pol ϵ proofreading-deficient cancer mutants likely have underlying MMR defect.

Figure 1. Effects on of the M630G active site mutation in human Pol ϵ on DNA synthesis and 3'-5' exonuclease activities

(A) Schematic of the 1189 aa human Pol ϵ construct used for *in vitro* experiments in this study and previously [35, 43]. The six conserved exonuclease (Exo) and polymerase (Pol) motifs are shown. The position of the M630G active site mutation is indicated. (B) 500 ng of purified M630G-Pol $\epsilon^{\text{exo}+}$ and M630G-Pol $\epsilon^{\text{exo}-}$ enzymes were run on 10% denaturing PAGE gels and stained by Coomassie. Molecular weight marker (MW) is shown with selected molecular weights indicated in kDa. (C) Primer extension assays of M630G-Pol ϵ . The relative DNA synthesis activities of wild type (M630) and mutant (M630G) human Pol ϵ enzymes were compared in both the exonuclease-proficient (exo^+) and – deficient (exo^-) backgrounds. Enzyme (1 nM) was incubated with all four dNTPs (25 μM each), 8 mM Mg^{2+} and a duplex DNA substrate containing a 19-mer deoxyribonucleotide primer hybridized to a complementary 45-mer template (100 nM). Reactions were carried out as described in the Materials and Methods. Reactions were performed at 37°C and started by the addition of enzyme. Aliquots were removed at 2, 5 and 10 minutes and products were resolved on a 12% denaturing acrylamide gel. Control substrate with no enzyme added is shown (–). (D) 3'-5' exonuclease assays of M630G-Pol ϵ . The relative 3'-5' exonuclease activities of wild type (M630) and mutant (M630G) human Pol ϵ were also compared in both the exonuclease-proficient (exo^+) and – deficient (exo^-) backgrounds. Enzyme was incubated with 8 mM Mg^{2+} and a ssDNA 18-mer deoxyribonucleotide substrate (100 nM) in the absence of dNTPs. Reactions were carried out as described in the Materials and Methods. Reactions were performed at 37°C and started with the addition of 0.5 nM enzyme. Aliquots were removed at 2, 5 and 10 minutes. Control substrate with no enzyme added is shown (–). Products were resolved on a 12% denaturing acrylamide gel.

Figure 2. Base substitution and frameshift error rates for human M630G-Pol $\epsilon^{\text{exo-}}$

(A) Error rates for base pair substitutions (BPS), overall frameshifts (FS), -1 and $+1$ frameshifts. Error rates were calculated as described [39]. Error rates are shown for the exonuclease-deficient (light gray bars) and $-$ proficient (gray bars) M630G active site mutant Pol ϵ . Included for comparison are error rates for the exonuclease-deficient M630 Pol ϵ that we characterized previously (black bars, [35]). (B) Fidelity of individual base pair substitutions for M630G-Pol $\epsilon^{\text{exo-}}$ and M630-Pol $\epsilon^{\text{exo-}}$. Error rates for each of the 12 possible mispairs for the exonuclease-deficient forms of M630G-Pol ϵ (gray bars, this study) and M630-Pol ϵ (black bars, [35]) were calculated as described [39]. (C) Individual *in vitro* replication errors made by the M630G-Pol $\epsilon^{\text{exo-}}$ enzyme in the forward mutation assay are indicated above the reference *lacZ* sequence. Single base deletions are shown as empty triangles and single base insertions are shown as filled triangles.

Figure 3. Effect of M630G-Pol $\epsilon^{\text{exo-}}$ expression in human cells on mutant frequency
 (A) HCT-116 mismatch repair-deficient cells were grown to 70% confluence in 100 mm dishes and then transfected with 3 μ g of the indicated vector DNA. After 48 hours, cells were harvested and lysed in buffer containing 1% Triton X-100. Cell extracts were probed by Western blot (WB) using antibodies against FLAG to detect the recombinant Pol ϵ , as well as against GFP and actin (*left*). Duplicate cell lysate samples were probed by WB using antibodies against the catalytic subunit of Pol ϵ (p261) to detect the total combined recombinant and endogenous Pol ϵ (*right*). Pol ϵ p261 levels were quantitated (NIH ImageJ) and are shown relative to levels in untransfected cells. (B) To measure mutant frequencies, cells were transfected as described in (A) with the indicated constructs, then grown for 48 hours and trypsinized. 500 cells were seeded in duplicate into media lacking 6-thioguanine (6-TG) and grown for 5–7 days to determine plating efficiency. 4.5×10^5 cells were seeded in triplicate into media containing 6-TG to select for *HPRT1*⁻ mutant cells and grown for 12–14 days. Colonies were then stained with crystal violet and counted. Mutant frequency was calculated by the following equation: (# 6-TG resistant colonies)/[(# 6-TG seeded cells) $\times$ (plating efficiency # scored colonies)/(plating efficiency # cells seeded)]. Colonies were defined as ≥ 50 cells. Average values and standard deviations from 3 independent experiments were calculated and plotted relative to HCT-116 cells transfected with empty vector. *P*-values are shown above each comparison where the difference was found to be significant (*, $p < 0.05$; **, $p < 0.005$).

Figure 4. Generation of cell lines stably expressing M630G-Pol ϵ^{exo-}

(A) Clones were evaluated for expression of M630G-Pol ϵ^{exo-} by Western blot. Cells were harvested and lysed in buffer containing 1% Triton X-100. Cell extracts were probed by Western blot using antibodies against FLAG, Pol ϵ catalytic subunit (α -p261), actin and GFP.

Table 1
***LacZ* mutant frequencies for human Pol ϵ alleles**

Mutant frequencies were measured using the *lacZ* forward mutation assay [39]. *LacZ* mutant frequencies were measured in this study for the Exo⁺-M630G and Exo⁻-M630G constructs. These experiments were performed identically to those in [35] in order to directly compare the results.

	Exo ⁺ -M630 ^a	Exo ⁻ -M630 ^a	Exo ⁺ -M630G	Exo ⁻ -M630G
Total No. of Plaques Counted	27,792	26,089	55,061	8,164
No. of <i>lacZ</i> Mutants	18	119	79	539
Mutant Frequency (x 10 ⁻⁴)	6.5	46	14	660
No. of <i>lacZ</i> Mutant Clones Sequenced	n.d.	119	79	88
Total No. of Sequenced Mutations	n.d.	123	79	99

^aValues taken from [35].

Table 2
Mutation rate measurements of cells stably expressing M630G-Pol ϵ^{exo-}

HCT-116 cells were untransfected (HCT-116 cells alone), stably transfected with the empty pIRES vector, or with vector containing the full-length M630G-Pol ϵ^{exo-} . Mutation rates were calculated using the MSS-MLE method by fluctuation analysis using 10 independent cultures for each cell line. Independent stable transfectant clones were analyzed for the M630G-Pol ϵ^{exo-} .

Cell Line	Mutation Rate ($\times 10^{-7}$)	95% CI	Fold Difference (rel. to vector alone)
M630G-Pol ϵ^{exo-} clone 1	280	(220–350)	4.1x
M630G-Pol ϵ^{exo-} clone 2	170	(130–220)	2.4x
HCT-116 cells + empty vector	70	(47–96)	1x
HCT-116 cells alone	59	(39–81)	0.8x

Table 3
Mutations sequenced in the *HPRT1* gene from cells expressing M630G-Pol $\epsilon^{\text{exo-}}$ and M630-Pol $\epsilon^{\text{exo+}}$

The HPRT1 ORF was amplified from independent 6- TG-resistant colonies expressing either the endogenous M630-Pol $\epsilon^{\text{exo+}}$ (WT-Pol ϵ) or M630G-Pol $\epsilon^{\text{exo-}}$ (M630G-Pol ϵ) and sequenced. In clones where an entire exon was deleted, the region surrounding that exon was amplified from genomic DNA and sequenced to identify possible intronic splice-site mutations.

Mutations	WT-Pol ϵ	M630G-Pol ϵ
Transversions		
A•T to T•A	0 (0%)	15 (45%)
A•T to C•G	0	0
G•C to C•G	0	0
G•C to T•A	3	0
Total	3 (9.7%)	15 (45%)
Transitions		
G•C to A•T	8 (26%)	6 (18%)
A•T to G•C	0	1
Total	8 (26%)	7 (21%)
Frameshifts		
+1	5	6
-1	12	2
Total	17 (55%)	8 (24%)
Other	3	3
Total Mutations	31	33