Human DNA polymerase N (POLN or pol δ) is the most recently discovered nuclear DNA polymerase in the human genome. It is an A-family DNA polymerase related to Escherichia coli pol I, human POLQ, and Drosophila Mus308. We report the first purification of the recombinant enzyme and examination of its biochemical properties, as a step toward understanding the functions of POLN. Unusual for an A-family DNA polymerase, POLN is a low fidelity enzyme incorporating T opposite template G with a frequency of 0.45 and G opposite template T with a frequency of 0.021. The frequency of misincorporation of T opposite template G is higher than any other known DNA polymerase. POLN has a processivity of DNA synthesis (1–100 nucleotides) similar to the exonuclease-deficient Klenow fragment of E. coli pol I, is inhibited by deoxyribonucleotides, and resistant to aphidicolin. The strand displacement activity of POLN was higher than exonuclease-deficient Klenow fragment. Furthermore, POLN can perform translesion synthesis past thymine glycol, a common endogenous and radiation-induced product of reactive oxygen species damage to DNA. Thymine glycol blocks DNA synthesis by most DNA polymerases, but POLN was particularly adept at efficient and accurate translesion synthesis past a 5S-thymine glycol.

The human genome contains 15 distinct known DNA polymerase genes, and these are classified into four families A, B, X, and Y based on their amino acid sequences (1). Human DNA polymerase N (POLN),2 the most recently discovered nuclear DNA polymerase, is an A-family enzyme with unknown function. The gene on chromosome 4p16.2 encodes a protein of 900 amino acid residues with a molecular mass of 100 kDa (2). The prototypical A-family DNA polymerase, Escherichia coli pol I is a high fidelity DNA polymerase that contributes to the maturation of Okazaki fragments during DNA replication and in gap-filling during base excision repair (BER), nucleotide excision repair (NER), and repair of DNA interstrand cross-links.

Human DNA polymerase N (POLN) is a Low Fidelity Enzyme Capable of Error-free Bypass of 5S-Thymine Glycol*

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Human POLQ, another A-family DNA polymerase, is similar to the Drosophila nuclear DNA polymerase Mus308 (3) in that it encodes both a DNA/RNA helicase domain and an A-family DNA polymerase domain (4, 5). By contrast, POLN has only the DNA polymerase domain. The POLN gene is encoded only in vertebrate genomes, but not in invertebrates or any lower eukaryotes. Possibly POLN has a role related to organ systems that are especially developed in vertebrates, such as the adaptive immune system or the brain. Expression studies of POLN are limited, but expression of the gene has been detected by Northern blotting in testes, heart and skeletal muscle tissue (2) and by expression sequence tagging in prostate, muscle, brain, and other organs. In cells from a human neuroblastoma patient, a chromosome fusion (1, 4) disrupting the DNA polymerase domain coding sequence of POLN was observed at diagnosis and at relapse. A (4, 17) fusion was detected at relapse only (6). It is possible that POLN might serve as a tumor suppressor in some cell types and that loss of its function could accelerate genome instability.

To understand the functions of human POLN, we examined the fidelity and the activity of DNA damage bypass. We find that POLN has very low fidelity and a remarkable insertion preference different from any other known human DNA polymerase. Further, POLN can perform accurate translesion synthesis past a common product of reactive oxygen species damage to DNA, the thymine glycol. The properties of POLN indicate a function distinct from that of POLQ.

EXPERIMENTAL PROCEDURES

Enzyme Purification—To construct a human POLN sequence construct for expression in E. coli, POLN with a short truncation of the C-terminal proline-rich tail was purified. This enzyme, previously referred to as POLN ΔP (2) shows no difference in activity from full-length POLN and is the same length as rodent POLN. This POLN and the corresponding D623A mutant were amplified by PCR from the plasmid pEGFP-C1 containing full-length wild type or D623A mutant POLN (2) using the primers 5′-CACCGAAAATATTAGGGCATTTGTTAGGC-3′ (for the 5′-end) and 5′-ATATAGAATTTCCACTTGTCTGCATC-GTCCTTTGATGCATGCGGTGCTGCATGCGACACTGG-3′ (for the 3′-end), and cloned into plasmid pENTR/D-TOPO (Invitrogen). After DNA sequencing, the cDNA was transferred into plasmid pDEST17 (Invitrogen) resulting in a protein tagged with six His residues at the N terminus (contributed by the pDEST17 vector), and a FLAG tag at the C terminus. The plasmid was transformed into an E. coli strain BL21.

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¶ The abbreviations used are: POLN, human DNA polymerase N; pol, polymerase; BER, base excision repair; NER, nucleotide excision repair; Tg, thymine glycol; CPD, cyclobutane pyrimidine dimer; 6-4 PP, (6-4) photoproduction; NEM, N-ethylmaleimide; Kf (exo-), exonuclease-deficient Klenow fragment of E. coli DNA polymerase I; DTT, dithiothreitol; BSA, bovine serum albumin.
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FIGURE 1. DNA polymerase activity of purified wild-type and mutant recombinant POLN. A, silver-stained gel showing molecular mass markers and 290 ng of purified POLN and POLN (D623A). Proteins were separated by electrophoresis on an 8% polyacrylamide gradient gel. B, 290 ng of POLN and POLN (D623A) were loaded onto a gel and immunoblotted with a 1:1000 dilution of POLN antibody (PA434). C, DNA polymerase activity was measured by extension of a 16-mer primer annealed to a 30-mer template. Samples from lanes 1–6 were incubated with 23 nM BSA for 10 min, incubation with 23 nM POLN for 0, 2.5, 5.0, and 10 min, and incubation with 23 nM POLN (D623A) for 0, 2.5, 5.0, and 10 min. Decreasing amounts of POLN (23 and 5.7 nM in lanes 1–6) or not annealed (lanes 7–12) were incubated with the same template but without dNTP for 10 min. Lane 1 contained no enzyme. E, TdT activity of POLN. 23 nM POLN was incubated with 300 fmol of 5'-32P-labeled 14-mer primer annealed to a 14-mer DNA (lanes 1–6) or not annealed (lanes 7–12), in the presence or absence of the indicated dNTPs (100 μM) for 10 min.

star (DE3), mutated for RNaseE (Invitrogen) and containing a plasmid (pRARE, Novagen) encoding 7 tRNAs for rare codons and the lysS+ gene. A single colony was incubated in 25 ml of Luria-Bertani medium (LB) containing 40 mM glucose, 50 μg/ml carbenicillin, and 50 μg/ml chloramphenicol at 37 °C. The culture was grown overnight and transferred into 500 ml of fresh LB containing 40 mM glucose, 50 μg/ml carbenicillin, and 50 μg/ml chloramphenicol. The culture was grown at 37 °C to an optical density of 600 nm of 0.5 and cooled down on ice for 30 min. The culture was incubated with 1 mM isopropyl-β-D-thiogalactopyranoside at 16 °C for 16 h. Incubation at 16 °C improved soluble protein expression relative to incubation at 37 °C. Cells were harvested by centrifugation (3000 × g for 10 min) and washed with ice-cold phosphate-buffered saline. After centrifugation (3000 × g for 10 min), 10 volumes of FLAG binding buffer (100 mM sodium phosphate pH 7.6, 10% glycerol, 5 mM EDTA, 0.1% Triton X-100, 0.1 mg/ml BSA, 100 mM desferrioxamine, and EDTA-free protease inhibitor mixture from Roche Applied Science was added to the pellet. The resuspended mixture was sonicated on ice (20 cycles of 10 s with a 20 s pause), and 0.25% polyethyleneimine was added and incubated for 15 min at 4 °C. DNA and debris were removed by centrifugation (15,000 × g for 15 min) and the supernatant incubated overnight at 4 °C with 200 μl of FLAG resin (Sigma). The resin was washed five times with 10 volumes of FLAG washing buffer (100 mM sodium phosphate, pH 7.6, 10% glycerol, 0.01% Nonidet P-40, and EDTA-free protease inhibitor mixture) and then three times with 10 volumes of TALON binding buffer (50 mM sodium phosphate, pH 7.0, 300 mM NaCl, 10% glycerol, 0.01% Nonidet P-40, and EDTA-free protease inhibitor mixture). The protein was eluted by incubating with 200 μg/ml FLAG peptide in 500 μl of TALON binding buffer for 1 h at 4 °C. The elution was incubated with 500 μl of TALON resin (BD Biosciences Clontech) for 1 h at 4 °C. The resin was washed five times with 10 volumes of TALON binding buffer and the protein eluted with 1 ml of TALON binding buffer containing 150 mM imidazole. Rabbit polyclonal antibody PA434, raised against an N-terminal 500-amino acid fragment of human POLN produced in E. coli (2) was used for immunoblotting.

DNA polymerase Q (POLQ) was as reported (5). RB69 (gp43) was expressed from a plasmid provided by W. Konigsberg and purified as described (7). Human DNA polymerase β (POLB) was provided by R. Sobol, and human DNA polymerase η (POLH) by C. Masutani and F. Hanaoka. Kf (exo-) and DNA polymerase θ (POLH) by C. Masutani and F. Hanaoka. Kf (exo-) and RB69 were obtained from Promega.

Oligonucleotide Substrates—Primer oligonucleotides were purchased from Bio-Synthesis or Sigma GenoSys, purified by HPLC or gel extraction, and 5’-labeled using polynucleotide kinase and [γ-32P]dATP. For 3’-labeling, the oligonucleotides
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were incubated with terminal deoxynucleotidyl transferase (TdT) and [α-32P]dATP. Oligonucleotides containing a single thymine-thymine CPD, thymine-thymine 6–4 photoproduct, or 5S-thymine glycol were synthesized as detailed earlier (8–10). The oligonucleotide containing a 1,2 d(GpG) cisplatin adduct was as described (11). The oligonucleotide containing a 1,2 d(GpG) cisplatin intranstrand adduct was as described (12). For strand displacement assays, a 60-mer oligonucleotide with either a 5′-phosphoryl or 5′-hydroxyl, 5′-CCCGAGGAAATCCGTTCATAGCTTGTTTCC-TGTCGAGGGCCGCGTGGT-GTTCCTTTTCTT was annealed together with the 24-mer oligomer to M13mp18GTGx ssDNA.

For assay of TdT activity, the 14-mer oligomer 5′-32P[AAGATGCTGAG]-TTCCTTTTCTT was assayed as a single strand or annealed to 5′-CTCGTCAGCATCTTTCT as a blunt end duplex.

DNA Polymerase Assays—A 5′-labeled 16-mer primer and a 30-mer template (sequences given above) were annealed at a molar ratio of 1:1 to detect DNA polymerase activity. The oligonucleotides were heated for 5 min at 65 °C and cooled down slowly for self-anneling. Standard reaction mixtures (10 μl) contained 20 mM Tris-HCl pH 8.8, 4% glycerol, 2 mM DTT, 80 μM dNTPs, the indicated concentrations of ddTTP, and 8 μg/ml poly(dA)/oligo(dT)10:1, sequence alignment of the motif 4 of A-family DNA polymerases.

Dark shading denotes the highly conserved aromatic residue that affects ddNTP sensitivity in bacterial DNA polymerases. The boxed residue (Lys or Arg in POLN, Ala or Thr in high fidelity of bacterial pol I, and Q in POLQ and Mus308) is a major determinant of fidelity in bacterial Pol I; see text for further details. POLN sequences: Hs, human; Mm, mouse; Dg, dog; Gl, chicken; Dr, zebrafish Danio rerio. POLQ sequences: Hs, human; Mm, mouse; Mus308, Drosophila melanogaster Mus308; CeMus1, Caenorhabditis elegans Mus1; Myctu, Mycobacterium tuberculosis pol I; EcPolI, E. coli pol I; Haein, Haemophilus influenzae pol I; Ricketts, Rickettsia prowazekii pol I; BstPolII, Bacillus stearothermophilus pol II; TaqPolI, Thermus aquaticus pol I; Trepon, Treponema pallidum pol I.

FIGURE 2. Biochemical properties of POLN on a poly(dA) template primed with oligo(dT) (10:1). A, aphidicolin resistance of POLN in comparison to E. coli Kf (exo-) pol I and T4 DNA polymerase. C, inhibition of POLN by ddNTPs. Reaction mixtures contained 10 μM dTTP, and 8 μg/ml poly(dA)/oligo(dT)10:1 template. D, sequence alignment of the motif 4 of A-family DNA polymerases. Dark shading denotes the highly conserved aromatic residue that affects ddNTP sensitivity in bacterial DNA polymerases. The boxed residue (Lys or Arg in POLN, Ala or Thr in high fidelity of bacterial pol I, and Q in POLQ and Mus308) is a major determinant of fidelity in bacterial Pol I; see text for further details. POLN sequences: Hs, human; Mm, mouse; Dg, dog; Gl, chicken; Dr, zebrafish Danio rerio. POLQ sequences: Hs, human; Mm, mouse. Mus308, Drosophila melanogaster Mus308; CeMus1, Caenorhabditis elegans Mus1; Myctu, Mycobacterium tuberculosis pol I; EcPolI, E. coli pol I; Haein, Haemophilus influenzae pol I; Ricketts, Rickettsia prowazekii pol I; BstPolII, Bacillus stearothermophilus pol II; TaqPolI, Thermus aquaticus pol I; Trepon, Treponema pallidum pol I.
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FIGURE 3. Processivity of human POLN. A, enzyme titration. Decreasing amounts of POLN (91, 45, 23, 11.5, and 5.7 pmol in lanes 2, 3, 4, 5, and 6, respectively) or Kf (exo-)(24, 12, 6, 3, and 1.5 pmol in lanes 8, 9, 10, 11, and 12, respectively) were incubated in reaction mixtures with 500 fmol of M13mp18GTGx single-stranded template annealed to 5'-32P-labeled 24-mer primer for 10 min. Lanes 1 and 7 contained no enzyme. B, trap. 500 fmol of 5'-32P-labeled primer templates and POLN (23 nm in lanes 2–8) of Kf (exo-) (6 nm in lanes 9–15) with different amounts of poly(dA)/oligo(dT)10:1 as trapping reagent (0, 0.125, 0.25, 0.5, 1.0, and 2.0 pmol in lanes 2–8 and 9–15, respectively). Lane 1 contained no enzyme. In lanes 8 and 15, 2.0 pmol of trapping reagent were added first, before enzymes were added. C, time course. POLN (23 nm in lanes 2–8) or Kf (exo-) (6 nm in lanes 9–15) was incubated with 500 fmol of 5'-32P-labeled primer templates for the indicated time periods.

To optimize conditions and assay sensitivity to aphidicolin (Fisher), N-ethylmaleimide (NEM) (Sigma) or ddTTP (2',3'-dideoxythymidine-5'-triphosphate, Amersham Biosciences), a poly(dA)-oligo(dT)10:1 was incubated with 500 fmol of the singly primed template annealed to 5'-32P-labeled 24-mer primer at a molar ratio of 2:1. POLN or Kf (exo-) was incubated with 500 fmol of the 30-mer template at 37 °C in 10 mM Kf (exo-) was incubated with 500 fmol of the 30-mer template at 37 °C in 10 mM Kf (exo-) was incubated with 500 fmol of the 30-mer template at 37 °C in 10 mM Kf (exo-) (24, 12, 6, 3, and 1.5 pmol in lanes 8, 9, 10, 11, and 12, respectively). The buffer for POLN contained 20 mM Tris-HCl, pH 8.8, 4% glycerol, 80 mM MgCl2, and 100 mM each dNTP. The buffer for POLB contained 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1.5 mM DTT, 200 µM/ml BSA, 10 mM MgCl2, and 100 µM each dNTP. The buffer for POLQ contained 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1.5 mM DTT, 200 µM/ml BSA, 10 mM MgCl2, and 100 µM each dNTP. Reactions were terminated by adding 10 µl of gel loading buffer and boiling at 95 °C for 3 min. Products were electrophoresed on a denaturing 10% polyacrylamide-7 M urea gel and exposed to BioMax MS film.

Translesion Synthesis Assays—The 5'-32P-labeled 16-mer primer annealed to the 30-mer template at a molar ratio of 1:1 was used as primer template. POLN, RB69 (gp43), POLB, pol I, Kf (exo-), POLH, or POLQ was incubated with 300 fmol of the primer template at 37 °C in 10 µl reactions. The buffer for POLN or Kf (exo-) was the same as given above. The buffer for RB69 (gp43) contained 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 1 mM DTT, 200 µg/ml BSA, 10 mM MgCl2, and 100 µM each dNTP. The buffer for POLB contained 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1.5 mM DTT, 200 µg/ml BSA, 200 µM EDTA, 10 mM MgCl2, and 100 µM each dNTP. The buffer for E. coli pol I contained 50 mM Tris-HCl pH 7.2, 100 µM DTT, 10 mM MgSO4, and 100 µM each dNTP.

Processivity Assay—Single-primed phagemid DNA templates were prepared by annealing the 5'-32P-labeled 24-mer primer to single-stranded M13mp18GTGx at a molar ratio of 2:1. POLN or Kf (exo-) was incubated with 500 fmol of the singly primed template at 37 °C in 10 µl of polymerase buffer. For Kf (exo-) the buffer contained 50 mM Tris-HCl pH 7.2, 100 µM DTT, 10 mM MgSO4, and 100 µM each dNTP. Reactions were terminated by adding 10 µl of gel loading buffer and boiling at 95 °C for 3 min. Products were electrophoresed on a denaturing 10% polyacrylamide-7 M urea gel and exposed to BioMax MS film.

Strand Displacement Assay—Substrates were prepared by mixing 5'-32P-labeled 16-mer primer, a downstream oligomer

plot of [dNTP]/ν versus [dNTP]. The nucleotide misincorporation ratio, fmax was determined by dividing (Vmax/Km) incorrect by (Vmax/Km) correct.

To optimize conditions and assay sensitivity to aphidicolin, N-ethylmaleimide (NEM) (Sigma) or ddTTP (2',3'-dideoxythymidine-5'-triphosphate, Amersham Biosciences), a poly(dA)-oligo(dT)10:1 was used instead of the 5'-labeled 16-mer primer annealed to the 30-mer template. Reaction mixtures (25 µl) contained 20 mM Tris-HCl pH 8.8 (unless otherwise indicated), 4% glycerol, 2 mM DTT, 80 µg/ml BSA, 8 mM Mg(C2H3O2)2 (unless otherwise indicated), 8 µg/ml of poly(dA)-oligo(dT)10:1, 10 µM dTTP, 1 µCi of [α-32P]dTTP, 23 mM POLN (unless otherwise indicated), and NaCl, aphidicolin, NEM or ddTTP as specified. After incubation at 37 °C for 20 min, reactions were stopped by adding 25 µl of 40 mM EDTA and placed on ice. A 10-µl aliquot of each mixture was spot-onto DE81 paper (Whatman), and washed three times with 0.5 M Na2HPO4 for 5 min and twice with ethanol. The paper was dried, and radioactivity was quantified with a Fuji Phosphor Imager. One unit of DNA polymerase activity was defined as the amount catalyzing the incorporation of 10 nmol of dTTP into poly(dA)/oligo(dT)10:1 template at 37 °C for 30 min.

Processivity Assay—Single-primed phagemid DNA templates were prepared by annealing the 5'-32P-labeled 24-mer primer to single-stranded M13mp18GTGx at a molar ratio of 2:1. POLN or Kf (exo-) was incubated with 500 fmol of the singly primed template at 37 °C in 10 µl of polymerase buffer. For Kf (exo-) the buffer contained 50 mM Tris-HCl pH 7.2, 100 µM DTT, 10 mM MgSO4, and 100 µM each dNTP. Reactions were terminated by adding 10 µl of gel loading buffer and boiling at 95 °C for 3 min. Products were electrophoresed on a denaturing 10% polyacrylamide-7 M urea gel and exposed to BioMax MS film.
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RESULTS

Purification and Biochemical Properties of Human DNA Polymerase N—The human POLN cDNA was expressed in E. coli and tagged with six His residues at the N terminus and a FLAG epitope at the C terminus. Protein sequentially purified on FLAG antibody beads and TALON resin migrated near the expected Mr of 102,000 (Fig. 1, A and B). POLN could extend DNA on a primed template whereas the active site mutant (D623A) showed no DNA polymerase activity (Fig. 1C). This residue is highly conserved in motif 3 of A-family DNA polymerases and is important in coordinating bivalent metal ions to interact with an incoming nucleotide (15). The DNA binding activity of POLN was not influenced by this substitution (data not shown). POLN lacks sequences corresponding to a 3′-5′ exonuclease domain, and no exonuclease activity was detected when enzyme and substrate were incubated without dNTPs (Fig. 1D). POLN showed limited TdT activity and was able to add one nucleotide, particularly dAMP, to the 3′-end of a double-stranded template, but not on a single-stranded template (Fig. 1E). Non-templated addition at a blunt end is a common feature of A-family DNA polymerases (16, 17).

The optimal reaction conditions for human POLN were determined using a poly(dA)/oligo(dT)_10 substrate. The optimal pH was 8.8, the same as POLQ (5) (data not shown). Like POLQ (4), the DNA polymerase activity of POLN was highest in reaction mixtures that contained no salt, and increasing concentrations of NaCl inhibited the activity with 50% inhibition at 200 mM. POLN required a bivalent cation, and the optimal concentration was 8 mM MgCl_2. MnCl_2 at 4 mM provided 29% of the optimal activity. There was no difference between Mg(C_2H_3O_2)_2 and MgCl_2 (not shown). Aphidicolin, which is an inhibitor of B-family DNA polymerases such as T4 DNA polymerase, had no inhibitory effect on POLN or Kf (exo-), respectively (Fig. 2A). Like Kf (exo-), POLN was relatively insensitive to NEM, whereas T4 DNA polymerase was sensitive (Fig. 2A). The specific activity of POLN with these optimal conditions was 125 units/mg.

A single aromatic residue in motif 4 of A-family DNA polymerases, either Phe or Tyr, is highly conserved and critical for ddNTPs selectivity (Fig. 2B). The specificity of POLN with these optimal conditions was 125 units/mg.

![Image](78x354 to 149x426)

**FIGURE 4. Nucleotide selectivities of POLN.** 23 nM POLN was incubated with 300 fmol of 5′-32P-labeled 16-mer primer annealed to a 30-mer DNA in the presence of one of the indicated dNTPs (100 μM) for 10 min. The first template base denoted by X was changed from A (lanes 1–4) to C (lanes 5–8), G (lanes 9–12), and T (lanes 13–16). Template bases are indicated on the left.

| Table 1 |
|-----------------|---------------|-------------|-------------|-----------------|
| DNA substrate | dNTP          | $K_\text{on}$ | $V_{\text{max}}$ | $V_{\text{max}}/K_\text{on}$ | $f_{\text{inc}}$ |
| Insertion opposite G | dATP         | 299 ± 6.0   | 0.24 ± 0.01 | 8.1 ± 0.2        | 8.2 × 10^{-3}  |
| 5′-ATG          | dCTP         | 8.2 ± 0.6   | 0.79 ± 0.22 | 995 ± 346        | 1             |
| Insertion opposite T | dATP         | 7.0 ± 1.4   | 2.2 ± 0.3   | 3210 ± 152       | 1             |
| 5′-ATG          | dCTP         | 538 ± 87    | 0.04 ± 0.01 | 67 ± 0.1         | 2.1 × 10^{-4}  |
| Insertion opposite A | dGTP         | 101 ± 10    | 0.69 ± 0.10 | 68.1 ± 3.0       | 2.1 × 10^{-2}  |
| POLH (insertion opposite T) | dATP         | 4.9 ± 0.8   | 2.1 ± 0.2   | 4320 ± 317       | 3.2 × 10^{-3}  |
| Insertion opposite C | dCTP         | 4.4 ± 0.5   | 3.6 ± 0.5   | 8300 ± 1980      | 1             |
| Insertion opposite C | dGTP         | 7.3 ± 0.6   | 1.2 ± 0.0   | 1680 ± 200       | 1             |
| Insertion opposite SS-Tg | dATP          | 11.8 ± 1.1  | 0.63 ± 0.02 | 535 ± 28.9       | 1             |
| 5′-ATG          | dGTP         | 313 ± 60    | 0.08 ± 0.01 | 2.5 ± 0.1        | 4.7 × 10^{-3}  |
| Insertion opposite SS-Tg | dGTP         | 26.9 ± 8.0  | 0.58 ± 0.21 | 210 ± 14         | 1             |
| Insertion opposite SS-Tg | dATP          | 247 ± 29    | 0.28 ± 0.06 | 11.2 ± 1.3       | 5.3 × 10^{-2}  |

![Image](166x354 to 235x426)

![Image](231x26 to 258x38)
replacing Phe<sup>667</sup> of Taq pol I or Phe<sup>762</sup> of E. coli pol I with Tyr decreases discrimination against ddNTPs (18). POLN has a Tyr<sup>682</sup> at the homologous position, and the DNA polymerase activity of POLN was inhibited by ddTTP on the poly(dA)-oligo(dT) template (Fig. 2C). Human POLQ with Tyr<sup>1882</sup> (4) or Drosophila Mus308 with Tyr<sup>1882</sup> (19) is also inhibited by ddNTP.

**Processivity of Human DNA Polymerase N**—To examine the processivity of POLN, a 5'-labeled 24-mer oligonucleotide was annealed to single-stranded circular M13 phage DNA and extension analyzed. The assay was performed in several ways to favor products resulting from a single binding event of POLN to the primer template. In an enzyme titration, POLN could elongate DNA chains as much as ~100–150 nt (Fig. 3A). The product distribution was retained in the presence of an excess amount of poly(dA)/oligo(dT)<sub>10:1</sub>, as a trapping agent (Fig. 3B), and products of >100 nt were observed after only 5 min (Fig. 3C). The pattern was similar to that produced by Kf (exo-) (Fig. 5, A–C), which also gives products of 1–100 nt (20). Thus, the processivity of human POLN is considerably greater than the processivity of 1–10 nt typically observed for X and Y family DNA polymerases.

**Unusual Fidelity Properties of POLN**—Prokaryotic A-family DNA polymerases have relatively high fidelities and contribute to error-free DNA synthesis. The human A-family DNA polymerase POLQ has low fidelity, however (5). We analyzed the fidelity of POLN using a 30-mer template with a 16-mer primer (Fig. 4). When the first template base was G, POLN frequently incorporated the incorrect nucleotide T when dTTP was the only deoxynucleotide present, (Fig. 4, lane 4). Incorporation of G across from template G and further extension past a noncomplementary G-T base pair was also observed (Fig. 4, lane 3). With template T, POLN usually incorporated A but also frequently incorporated the incorrect base G (Fig. 4, lane 7). When the first template base was A, POLN usually incorporated the correct base T but also incorporated an extra incorrect T opposite the following template T (Fig. 4, lane 12). With template C, POLN frequently incorporated the correct choice G opposite the first template C, and extended further by incorporating G opposite the following template T (Fig. 4, lane 15). These experiments indicated that POLN catalyzes considerable misincorporation, particularly by incorporating T opposite G and G opposite T.

To examine the fidelity of POLN, kinetic parameters were determined. We examined the template (first template base G) on which POLN appeared to incorporate incorrect bases most frequently (Fig. 4). Measurements were obtained with other incoming nucleotides, and from the ratio $V_{\text{max}}/K_m$ (13), a misincorporation frequency (f<sub>inc</sub>) was calculated. Compared with a value of 1.0 for correct utilization of dCTP opposite template G, these were $8.2 \times 10^{-3}$.

![FIGURE 5](image_url)

**Translesion synthesis by human POLN.** Increasing amounts of RB69 (gp43) (2.4, 4.8, and 9.5 pm in lanes 2–4), POLB (75, 150, and 300 pm in lanes 6–8), pol I (2.3, 4.6, and 9.2 pm in lanes 10–12), Kf (exo-) (0.23, 0.46, and 0.92 pm in lanes 14–16), POLH (0.27, 0.54, and 1.08 nm in lanes 18–20), POLQ (2.8, 5.7, and 11.3 nm in lanes 22–24) or POLN (5.7, 11.3, and 23 nm in lanes 26–28) were incubated with the 5'-<sup>32</sup>P-labeled primer templates indicated beside each panel at 37 °C for 10 min. Lanes 1, 5, 9, 13, 17, 21, and 25 contained no enzyme. A, undamaged control; B, 1,2-di(GpG) cisplatin adduct; C, AP analog; D, 5S-Tg; E, 5R-Tg; F, T-T CPD; G, T-T 6–4 PP. In part A the percent (%) extension of the primer is shown below each lane.
for dATP, $3.6 \times 10^{-2}$ for dGTP and $4.5 \times 10^{-1}$ for dTTP (Table 1). These values are about 100 times higher than the misincorporation frequency of $10^{-5}$ to $10^{-4}$ found for Kf (exo-) or Taq pol I (21–23) and are closer to the values found for Y-family DNA polymerases (24). The $f_{inc}$ value for misincorporation of T opposite template G was especially remarkable.

To further compare the fidelity with that of other DNA polymerases, we examined incorporation with first template base T. For POLH and Kf (exo-), the $K_m$ and $V_{max}$ values for incorporation of A opposite template T were similar to values previously reported (5, 14, 25) and were similar, within a few fold, to the corresponding values for human POLN (Table 1). Compared with a value of 1.0 for correct utilization of dATP opposite template T, the $f_{inc}$ values were $2.1 \times 10^{-4}$ for dCTP, $2.1 \times 10^{-2}$ for dGTP and $3.2 \times 10^{-3}$ for dTTP. Among these values, G misincorporation was highest and was similar to $2.0 \times 10^{-2}$ found for POLQ or $7.9 \times 10^{-2}$ for POLH (14). From these results, we conclude that human POLN is a low fidelity DNA polymerase.

POLN Efficiently Bypasses a Thymine Glycol in DNA and Shows Strand Displacement Activity—Some low fidelity DNA polymerases can perform translesion synthesis past certain types of DNA damage. To examine the activity of human POLN in this respect, a 5'-32P-labeled primer with a 3'-terminus located just before the lesion was annealed to templates containing either no damage, a 1,2 (dGpG) adduct produced by cis-diaminedichloroplatinum (II) (cisplatin), an abasic (AP) site, a 5'- or 5R-diastereoisomer of thymine glycol (Tg), a cyclobutane pyrimidine dimer (CPD), or a 6–4 photoproduct (6–4 PP). For comparison, the DNA polymerases RB69 (gp43), POLB, E. coli pol I (pol I), Kf (exo-), POLH, and POLQ were also tested on the same substrates. Quantities of DNA polymerases were chosen that gave rise to similar total amounts of products on undamaged substrate. All DNA polymerases except POLB could synthesize DNA products of up to 30 nt in length on the undamaged template (Fig. 5A). With a higher concentration (9.5 nM) of POLB, products up to 30 mer could be synthesized and most of the template was extended (not shown). RB69 (gp43), a highly processive
enzyme, produced not only 30-mer products but also shorter products, because of its robust 3’-5’ exonuclease activity (Fig. 5A, lanes 1–4).

POLN, POLQ (5), and most of the other DNA polymerases tested could not insert a nucleotide opposite the cisplatin adduct. POLH could incorporate one nucleotide opposite the first G of the adduct, and less frequently incorporated a base opposite the second G (26) (Fig. 5, lanes 17–20). POLB could not extend primers from a cisplatin-GG adduct at the concentration used here, though it can bypass the adduct when a 105-fold higher concentration of POLB is used (27).

It is notable that POLN could not bypass an AP site, but the closely related POLQ can do so (Fig. 5C, lanes 21–28) (5). As reported previously (26), POLH was able to bypass an AP site, but synthesis usually stopped after incorporation of one nucleotide opposite the AP site (26). It was efficient (14), with some incorporation the first template base was 5’-C, G, or T (Fig. 5, lanes 9–12). When the first template base was 5R-Tg, or an undamaged T, POLN usually incorporated the correct base A but sometimes G (Fig. 6, lane 17). As determined by measuring kinetic parameters, the relative efficiency of incorporation of A opposite a 5S-Tg compared with a non-damaged template T was 16.7%; for a 5R-Tg, was 6.5% (Table 1). Incorporation of G opposite 5S-thymine glycol was considerably less efficient (Table 1). These results indicated that POLN can bypass 5S-Tg well and with a higher fidelity than on an undamaged T template. Thus, POLN efficiently carries out both the error-free insertion and extension steps for bypass of 5S-Tg.

Strand Displacement Activity of POLN—Pol I of E. coli has strand displacement and 5’-3’ exonuclease activities that are used during DNA repair and Okazaki fragment maturation. The strand displacement activity of POLN was tested on substrates containing a nick or a 1-nt gap. Strand displacement synthesis is represented by products of >16 nt. POLN was very efficient at DNA synthesis on a nicked template and generated less stalled product than the other DNA polymerases tested (Fig. 7A). RB69 (gp43) could hardly synthesize DNA on this template (Fig. 7A, lanes 1–4). POLN had weak strand displacement activity as reported (29) (Fig. 7A, lanes 5–8). Pol I and Kf (exo-) were able to catalyze strand displacement DNA synthesis; the products of pol I with 5’-3’ exonuclease (nick translation) activity were more abundant than those produced by Kf (exo-) (Fig. 7A, lanes 9–16). As reported previously (30), POLN catalyzed strand displacement (Fig. 7A, lanes 17–20). POLN also could synthesize DNA efficiently on a template with a single nucleotide gap (Fig. 7B, lanes 21–24). POLB, which efficiently seals a single nt gap during short patch BER (31) produced more products than on the nicked template (Fig. 7B,
lanes 5–8). These experiments indicate that POLN possesses considerable strand displacement activity.

To examine the processivity of the strand displacement activity, a 5'-labeled 24-mer primer was annealed to an M13mp18GTGx ssDNA template. Both POLN and Kf (exo-) synthesized products (Fig. 7C, lanes 1–8) similar to those shown in Fig. 3. When a downstream 60-mer oligonucleotide was annealed to generate a nick, Kf (exo-) was strongly inhibited by either 5'-phosphate or 5'-hydroxyl terminal duplex DNA (Fig. 7C, lanes 13–16 and 21–24). POLN could synthesize DNA by displacing the duplex DNA 10 bp or more (Fig. 7C, lanes 9–12 and 17–20). The use of a 60-mer downstream oligonucleotide (rather than the 14-mer used in Fig. 7A) revealed that POLN has a much stronger strand displacement activity than Kf (exo-). Although POLN does not have an intact 5'-3' exonuclease motif, the possibility of 5'-3' exonuclease activity was checked by incubating POLN under the same conditions but with a 3'-labeled 14-mer downstream oligomer. POLN gave products for the nicked substrate as efficiently as for the substrate without downstream oligomer (Fig. 7D, lanes 1–4), but did not degrade the 14-mer downstream oligomer (Fig. 7D, lanes 5 and 6). These results indicate that human POLN catalyzes efficient strand displacement synthesis.

DISCUSSION

Understanding the individual functions of the many different DNA polymerases in vertebrate cells is a major challenge that increases in complexity with the discovery of each DNA polymerase. The present work characterizes the enzymatic activity of the most recently discovered of these DNA polymerases, DNA POLN. Our initial approach has been to gain information on what types of reactions POLN can perform, which may provide clues as to its likely function. The properties of POLN that distinguish it from other DNA polymer-
ases in mammalian cells are its unusual fidelity properties, its ability to efficiently and accurately bypass thymine glycol, and its high capacity for strand displacement.

**Unique Properties of POLN in Fidelity and Ability to Bypass Thymine Glycol**—The most remarkable feature of DNA polymerase POLN with respect to fidelity is its ability to efficiently incorporate T opposite template G (~45% as well as C is incorporated opposite template G). This is a unique property not only among A-family DNA polymerases, but also among all other known DNA polymerases. The Y-family DNA polymerase POLI has the unique fidelity characteristic of efficiently catalyzing the inverse event, incorporation of G opposite template T (32). Most errors with POLI occur at template T as a result of analyzing the inverse event, incorporation of G opposite templatease POLI has the unique fidelity characteristic of efficiently catalyzing the inverse event, incorporation of G opposite template T (32). Most errors with POLI occur at template T as a result of analyzing the inverse event, incorporation of G opposite template T (32). For POLN, we found no significant difference among the $V_{\text{max}}/K_m$ values with different template bases (Table 1), and so misincorporation by POLN probably uses a mechanism different from POLI. POLN also is relatively efficient at incorporation of G opposite T. Although there might be biological scenarios where incorporation of T opposite G would be desirable, it seems more likely at present that the unusual misincorporation properties of POLN are related to its ability to bypass one or more specific forms of DNA damage.

In this respect, the preferential incorporation by POLN of A opposite Tg and its efficient extension from this lesion is notable. Tg lesions are a strong block to many DNA polymerases *in vitro* and *in vivo* (34). Neither isomer of thymine glycol can form a stable base pair with any nucleobase on the complementary strand (35), and DNA synthesis terminates after insertion of A opposite Tg. For example, in our experiments, DNA synthesis by the B-family DNA polymerase from RB69 (gp43) was blocked by either Tg isomer. In this respect, POLN is remarkable in that it is able to “correctly” incorporate an A opposite a Tg lesion, and then efficiently extend it. POLN misincorporates G opposite 5S-Tg ($f_{\text{inc}} = 4.7 \times 10^{-3}$) even more rarely than it misincorporates G across from an undamaged T ($f_{\text{inc}} = 2.1 \times 10^{-2}$) or 5R-Tg ($f_{\text{inc}} = 5.3 \times 10^{-2}$). Human POLH also can replicate through the 5R-Tg lesion efficiently, but the $f_{\text{inc}}$ value for dGMP is 1.2 $\times$ 10$^{-1}$ and is higher than the value for normal template T ($f_{\text{inc}} = 7.9 \times 10^{-2}$) (14). POLH is in this respect better adapted for error-free bypass of CPD (14).

Most repair of Tg in mammalian cells proceeds by BER, initiated by NEIL1 and NTH1 DNA glycosylases (36). HeLa cell extracts or mouse ES cell nuclear extracts remove 5R-Tg considerably faster than 5S-Tg, and so the latter lesion might be more frequently encountered by a DNA replication fork (36, 37). Use of POLN to bypass 5S-Tg with comparatively high efficiency and accuracy of bypass would be consistent with the low mutation frequency induced by Tg (38, 39).

**POLN Has Remarkable Strand Displacement Activity**—POLN can perform efficient strand displacement past a nick or a gap and gives rise to an amount of product similar to that on undamaged template (Figs. 5A, 7A, and 7B). By contrast, strand displacement by POLB alone is weak (Fig. 7A), although protein factors such as FEN1, PARP1, the RAD9-RAD1-HUS1 complex, or WRN helicase can enhance strand displacement DNA synthesis of POLB (29, 40–42). Mouse cell extracts lacking POLB still have residual BER activity, even in the presence of aphidicolin, which inhibits DNA polymerase δ and ε, back-up DNA polymerases activity for BER (43). As POLN is aphidicolin-resistant and has strand displacement activity, it may be a good candidate for a further back-up activity in “long patch” BER. A role for POLN in error-prone BER at G-C base pairs in hypermutation of immunoglobulin variable genes (44) is also conceivable.

**Mechanism of the Unique Properties of POLN**—The distinguishing fidelity and bypass properties of POLN must be related to specific features of its protein sequence and structure. Although further experimentation and structural work will be necessary to gain further insight into this, we briefly point out several features here.

In the “fingers” subdomain of *E. coli* pol I and Taq pol I, the O-helix (motif 4), has numerous contacts with the incoming dNTP that contribute to proper positioning in the active site and to accuracy. Mutation of Thr$^{644}$ to Arg in Taq pol I or mutation of the equivalent Ala$^{779}$ residue to Arg in exonuclease-deficient *E. coli* pol I creates a highly error-prone DNA polymerase (45, 46). These changes may stabilize the enzyme in the closed conformation, favoring misincorporation (47). The corresponding residue in human POLN is Lys$^{679}$, and it is Lys or Arg in POLN of other vertebrates (see Fig. 2D), so it is possible that this residue is a major determinant of fidelity. A further point of interest is that POLN has an insertion in the tip of the thumb subdomain, shorter than the 22-residue insert of POLQ (5), but in a region of the protein that is important for DNA binding (22, 48, 49).

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