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DNA Polymerase λ, a Novel DNA Repair Enzyme in Human Cells*

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DNA polymerase lambda (pol λ) is a novel family X DNA polymerase that has been suggested to play a role in meiotic recombination and DNA repair. The recent demonstration of an intrinsic 5′-deoxyribose-5-phosphate lyase activity in pol λ supports a function of this enzyme in base excision repair. However, the biochemical properties of the polymerization activity of this enzyme are still largely unknown. We have cloned and purified human pol λ to homogeneity in a soluble and active form, and we present here a biochemical description of its polymerization features. In support of a role in DNA repair, pol λ inserts nucleotides in a DNA template-dependent manner and is processive in small gaps containing a 5′-phosphate group. These properties, together with its nucleotide insertion fidelity parameters and lack of proofreading activity, indicate that pol λ is a novel β-like DNA polymerase. However, the high affinity of pol λ for dNTPs (37-fold over pol β) is consistent with its possible involvement in DNA transactions occurring under low cellular levels of dNTPs. This suggests that, despite their similarities, pol β and pol λ have nonredundant *in vivo* functions.

All known DNA polymerases are believed to share a remarkably high functional and structural similarity (1, 2). However, each of these enzymes possesses unique features that are crucial to cope with the different DNA transactions encountered while conducting DNA synthesis *in vivo*. Thus, in addition to the extensive DNA polymerization performed by replicative DNA polymerases, a large and growing number of enzymes have been found to be specialized in largely different and sometimes surprising types of synthesis (3–6).

Among these enzymes is mammalian pol[1] β, a member of the family X DNA polymerases. Polymerase β has been studied extensively and is now considered the paradigm of a repair DNA polymerase: it is ubiquitously expressed (7), and its biochemical features suggest that its *in vivo* role likely involves filling short gaps of DNA (8). Since its discovery, pol β has been suggested to play a role in DNA synthesis associated with DNA repair processes in the nucleus of mammalian cells. Indeed, it is accepted today that its polymerization features, combined with its dRP lyase activity, make pol β a crucial protein for base excision repair (9–11).

In addition to pol β, all other enzymes belonging to family X (12) are small, monomeric enzymes that can be found in all realms of life, including archaea, eubacteria, eukaryotes, and viruses (13). The first family X enzyme to be identified in mammalian cells was terminal deoxynucleotidyl transferase, a template-independent enzyme that adds nucleotides at the junctions of rearranged Ig genes (14). In addition to terminal deoxynucleotidyl transferase and mammalian pol β, a large number of family X enzymes have been now characterized in different organisms, including yeast pol IV (15, 16); pol κ/Trf 4 (17), recently renamed as pol σ (18); pol μ (19); and pol λ (20).

Polymerase λ is a nuclear enzyme that has been identified in mammals as well as different high eukaryotes including other vertebrates and plants such as *Arabidopsis thaliana* (20–22). In addition to its 32% amino acid identity with pol β, sequence comparison and three-dimensional structure modeling predict that pol λ contains all four pol β structural subdomains, named fingers, palm, thumb, and 8 kDa (20). However, unlike pol β, pol λ has a BRCT domain at its N terminus, which likely takes part in protein-protein or protein-DNA interactions (20–22). Northern blot analysis reflects that murine and human pol λ mRNA is highly abundant in testis (20, 22). Moreover, predominant expression of murine pol λ in pachytene spermatocytes has led to the hypothesis that it plays a role in DNA repair synthesis coupled to meiotic recombination (20). Besides having an intrinsic DNA polymerase activity, and the recent demonstration of an intrinsic dRP lyase activity (23), little is known about the biochemical features of pol λ. This is mainly because previous studies were limited by the lack of purified protein. Here, we describe the purification of human pol λ and its basic *in vitro* polymerization features and discuss novel insights into its cellular function.

EXPERIMENTAL PROCEDURES

Materials

Synthetic oligonucleotides purified by PAGE were obtained from Invitrogen. Ultrapure dNTPs, ultrapure ddNTPs, activated calf thymus DNA, [γ-^{32}P]ATP, [α-^{32}P]dATP, and [α-^{32}P]dTTP (3,000 Ci/mmol) were from Amersham Biosciences. *Taq* DNA polymerase was from PerkinElmer Life Sciences. T4 polynucleotide kinase was from Pro-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ131890.

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[1] The abbreviations used are: pol, DNA polymerase; BRCT, BRCA1 C terminus; BSA, bovine serum albumin; dd, dideoxy; dRP, 5′-deoxyribose 5-phosphate; DTT, dithiothreitol; EST, expressed sequence tag; HPRT, hypoxanthine guanine phosphoribosyl transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; PC, phosphocellulose.

mega. Purified human pol β was a generous gift from Dr. S. H. Wilson (NIEHS).

Cloning of the Human pol λ cDNA

Cloning of the human *POLL* gene was initiated by the identification, in the public data base dbEST/GenBank, of a collection of ESTs that showed a high similarity with the mouse pol λ cDNA sequence, previously obtained in our laboratory (20). The several ESTs identified, with accession numbers AA742404, AA922738, AI091150, AA989195, W69567, AI123218, AI199486, AA576526, W69888, and T81701, corresponded to the 3′-untranslated region of murine pol λ cDNA, with the exception of EST H11886, which contained part of the coding region. Human pol λ cDNA was obtained from human placenta by PCR amplification. A first fragment (1419 bp long), spanning position 1107–2525 of the complete cDNA sequence (2678 nucleotides), was obtained by specific PCR using primers derived from the ESTs described above. The 3′-terminal segment, from 2526 to 2678, was deduced from the consensus of the ESTs. A second fragment (996 bp long) was obtained by semispecific PCR, using a sense primer derived from the murine sequence close to the initiation codon and an antisense primer derived from the first PCR fragment (1419 bp long), which contained the coding sequence corresponding to positions 380–1375 of human pol λ cDNA. The cDNA sequence was completed by 5′-rapid amplification of cDNA ends, a 504-bp long fragment that contained the untranslated 5′-region, and the initial portion of the coding region.

Therefore, the complete cDNA of human pol λ contains a total of 2678 bp, with a 5′-untranslated region of 371 bp (1–371), a coding sequence spanning 1728 bp (372–2099), and a 3′-untranslated region of 579 bp (2100–2678).

Overproduction and Purification of Human pol λ Protein

The complete coding sequence corresponding to pol λ was cloned in the *Bam*HI-*Eco*RI sites of the bacterial expression vector pRSET-B (Invitrogen), which allows the expression of recombinant proteins as fusions with a multifunctional leader peptide containing a hexahistidyl sequence for purification on Ni^{2+}-affinity resins (24). Expression of pol λ was carried out in the *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL (Stratagene), with extra copies of the *argU*, *ileY*, and *leuW* tRNA genes. Expression of pol λ protein was induced by the addition of 1 mM IPTG to 3 liters of log phase *E. coli* cells grown at 28 °C in LB to an A_{600} of 0.5. After 20 min of induction, rifampicin was added to a final concentration of 120 μg/ml, and cells were incubated at 28 °C for 2 h. Subsequently, the cultured cells were harvested, and the pelleted cells were weighted (7.5 g) and frozen (−20 °C). Just before purification, which was carried out at 4 °C, frozen cells were thawed and ground with 5.5-fold their weight of alumina (Sigma) for 20 min, in the presence (per g of cells) of 1 volume of buffer A (50 mM Tris, pH 7.5, 10% glycerol, 0.5 mM EDTA, 1 mM DTT) supplemented with 1 M NaCl. The cell lysates were collected with 4 volumes of buffer A and 1 M NaCl. Cell debris and alumina were discarded after a 5-min centrifugation at 1,500 × *g*. Insoluble material was pelleted by a 20-min centrifugation at 12,000 × *g*. DNA was precipitated with 0.3% polyethyleneimine (10% stock solution in water, pH 7.5) and sedimented by centrifugation for 10 min at 12,000 × *g*. The supernatant was diluted to a final concentration of 250 mM NaCl with buffer A and precipitated with ammonium sulfate to 65% saturation to obtain a polyethyleneimine-free protein pellet. This pellet was resuspended in 100 ml of buffer A and 50 mM NaCl and loaded into a 3-ml PC column equilibrated previously in this buffer. After exhaustive washing with buffer A and 100 mM NaCl, pol λ was eluted with buffer A and 200 mM NaCl. The eluate containing pol λ was diluted with an equal volume of buffer B (50 mM Tris, pH 7.5, 10% glycerol) and loaded into a second PC column (1 ml), equilibrated previously in buffer A and 100 mM NaCl. The column was washed with buffer B supplemented with 100 mM NaCl, and the protein eluted with native binding buffer (20 mM phosphate buffer, pH 7.8, 500 mM NaCl; Invitrogen). The eluate was loaded into a Ni-NTA-agarose (Invitrogen) column equilibrated with native binding buffer. After several washing steps with native binding buffer containing increasing concentration of imidazole, pol λ was eluted at 400 mM imidazole. Polymerase λ-containing fractions were collected, 5-fold diluted with buffer A, bound to a third PC column (1 ml), and eluted with buffer A containing 500 mM NaCl. This fraction contains highly purified (>99%) human pol λ. Protein concentration was estimated by densitometry of Coomassie Blue-stained 10% SDS-polyacrylamide gels, using standards of known concentration. Under these conditions, the yield was 25 μg of purified pol λ/g of *E. coli* cells. The final fraction, adjusted to 50% (v/v) glycerol and supplemented with 0.1 mg/ml BSA, was stored at −70 °C. When indicated, the pure fraction was subjected to sedimentation in a 15–30% glycerol gradient. 100 μg of protein was loaded onto a 5-ml glycerol gradient containing 20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1 mM DTT, and 1 mM EDTA and centrifuged at 62,000 rpm (Beckman SW.50 rotor) for 26 h at 4 °C. Individual 200-μl fractions were collected from the top of the tube, examined in Coomassie Blue-stained 0.1% SDS-polyacrylamide gels, and tested for DNA polymerase activity on activated DNA.

Quantitative PCR Analysis of mRNA Expression

Quantitative PCR analysis was performed at standard conditions (LightCycler, Roche Biochemicals), using manufacturer's protocol (LightCycler Fast Start DNA Master SYBR Green I). A commercial panel of human tissue cDNA libraries (CLONTECH) was used as prenormalized cDNA templates. Each template cDNA was diluted 5-fold, and the resulting aliquot was used as a 5-fold stock for PCR. Amplification parameters were as follows: 94 °C, 5 s; 60 °C, 5 s; 72 °C, 10 s; 45 cycles (HPRT); 94 °C, 5 s; 62 °C, 5 s; 72 °C, 10 s; 45 cycles (human pol λ and human pol β). The primers, provided at 5 μM, were: HPRT952as, 5′-AACAACAATCCGCCCAAAG; HPRT554s, 5′-ATGGTCAAGGTCGCAAGCT (human HPRT); hpolβ700s, 5′-CCCATCCCAGCTTCACTTC; hpolβ1080as, 5′-CCCGGTATTTCCACTGGAT (human pol β); 3NTs, 5′-CCACTGCCCCTCGAAGAAT; 3NTas, 5′-TCCCAGCACCACCAGCTGC (human pol λ). After normalization of the various template cDNAs with the human HPRT primers, quantitative PCR of pol λ and pol β expression was carried out separately. Standard curves were made for each experiment, using series of sample dilutions. Relative expression differences were calculated by second derivate maximum analysis using the software package of LightCycler (Roche Diagnostics).

3′ → 5′ Exonuclease Assays

The incubation mixture, in 20 μl, contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 50 nM pol λ, and 1.5 nM single stranded labeled P1 or P1/T6T hybrid. Reactions were incubated at 37 °C for 20 min and stopped by adding 10 mM EDTA. The 3′ → 5′ exonucleolysis, expected to produce a degradation ladder of the labeled P1 primer, was analyzed by 8 M urea and 20% PAGE and autoradiography.

DNA Polymerization on Activated DNA and Poly(dA)·Oligo(dT)

The standard assay (25 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 13.3 nM [α-^{32}P] dTTP or dATP, and 625 ng of activated calf thymus DNA or 100 ng of poly(dA)·oligo(dT). When indicated, reaction components were added or omitted. Reactions were initiated by adding 3 nM pol λ or pol β and incubated for 10 min at 37 °C. After incubation, the reaction was stopped by adding 10 mM EDTA, and the samples were filtered through Sephadex G-50 spin columns. Polymerization activity was proportional to the amount of radioactivity present in the excluded volume, determined by counting Cerenkov radiation.

DNA Polymerization Assay on Defined DNA Molecules

Oligonucleotide P1, 5′-GATCACAGTGAGTAC, was used as the primer strand. Oligonucleotides T6A (5′-TCTATAGTACTCACTGTGATC), T6C (5′-TCTATCGTACTCACTGTGATC), T6G (5′-TCTATGGTACTCACTGTGATC), T6T (5′-TCTATTGTACTCACTGTGATC), and T18 (5′-ACTGGCCGTCGTTCTATTGTACTCACTGTGATC) were used as template strands. Oligonucleotides D1 (5′-AACGACGGCCAGT) and D1P (D1 with a 5′-phosphate), complementary to the 13 first 5′-nucleotides of T18, were used as downstream oligonucleotides to construct 5 nucleotide gaps. Oligonucleotide P1 (1 mM) was labeled at its 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase. This labeled oligonucleotide was then hybridized to one (template) or two (template and downstream) oligonucleotides in the presence of 50 mM Tris-HCl, pH 7.5, and 0.3 M NaCl. The incubation mixture (20 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 50 nM pol λ or pol β, and 1.5 nM hybrid, indicated in each case. Reactions were started by the addition of the indicated concentration of one or each of the four dNTPs and incubated at 37 °C for the indicated times. After incubation, reactions were stopped by adding 10 mM EDTA and analyzed by 8 M urea and 20% PAGE and autoradiography.

Kinetic Analysis of DNA Polymerization

Steady-state Conditions—DNA polymerization assays were performed as described above, using activated DNA and different dNTP concentrations. Measured enzyme velocity (fmol/min) was plotted as a

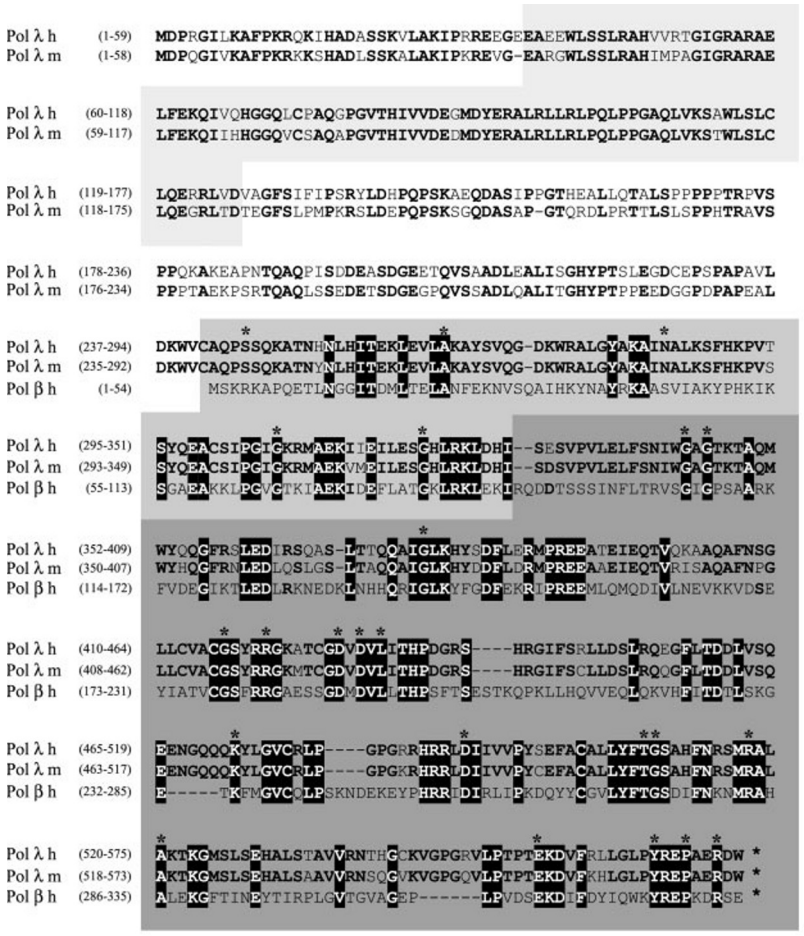

FIG. 1. **Multiple amino acid alignment of human and mouse orthologs of pol λ with human pol β.** *Numbers* indicate the amino acid position relative to the N terminus of each DNA polymerase. Invariant residues in the three enzymes aligned are indicated in *white letters* over a *black background*. Other amino acid identities with respect to human pol λ sequence are indicated in *bold letters*. Amino acids 36–126 (human pol λ) and 35–125 (murine pol λ (20)) are predicted to form a BRCT domain (*light gray*). Amino acids 241–575 (human pol λ) and 239–573 (murine pol λ (20)) form a conserved β-core, including an 8-kDa domain (*gray*) and a 31-kDa polymerization domain (*dark gray*). The 23 amino acid residues that are invariant among all family X DNA polymerases (34) are indicated by an *asterisk* at the *top* of the alignment.

function of dNTP concentration. The plotted data were fitted by a nonlinear regression curve to the Michaelis-Menten equation

$$V = (V_{max}[\text{dNTP}])/(K_m + [\text{dNTP}]) \quad \text{(Eq. 1)}$$

using KaleidaGraph software (Synergy Software, www.synergy.com). V_{max} and $K_{m(app)}$ values were obtained from the fitted curves.

Single Turnover Analysis—Oligonucleotide P1 hybridized to oligonucleotide T6T was used as DNA substrate. Reactions (20 μl) were performed as described above using 1.5 nM substrate and 20 nM pol λ or pol β. For each dNTP concentration, the amount of product formed with time was fit to a single exponential,

$$[\text{product}] = A(1 - e^{-k_{obs}t}) \quad \text{(Eq. 2)}$$

where A is the amplitude of the exponential and k_{obs} the exponential rate constant. The obtained single exponential rate constants were plotted as a function of substrate concentration and fit to a hyperbola,

$$k_{obs} = (k_{pol}[\text{dNTP}])/(K_d + [\text{dNTP}]) \quad \text{(Eq. 3)}$$

to derive K_d and k_{pol} values, the equilibrium dissociation constant for dNTP and intrinsic rate of insertion, respectively.

Short Gap Fidelity Assay

Gap filling reactions mixtures (20 μl) contained 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 1 mM DTT; 0.1 mg/ml BSA; 4% glycerol; 0.5 mM each dATP, dTTP, dGTP, and dCTP; 1.6 nM gapped M13mp2 DNA; 50 nM pol λ; and 400 units of T4 DNA ligase. After incubation at 37 °C for 60 min, EDTA was added to 15 mM, and reaction products were resolved by electrophoresis in a neutral 0.8% agarose gel. Covalently closed, circular DNA products were electroeluted from gel slices, recovered by ethanol precipitation, introduced into *E. coli* MC1061 by electroporation, and plated. After scoring revertant and total plaques, the DNA of revertants was sequenced to define the sequence change responsible for the change of phenotype.

RESULTS

Structural Features of Human pol λ—The human pol λ cDNA was cloned on the basis of its sequence homology with murine pol λ (*POLL* gene (20); see "Experimental Procedures"). The resulting cDNA is 2678 bp in length, with a 371-bp 5′-untranslated region, a 1728-bp coding sequence, and a 579-bp 3′-untranslated region, and it exhibits 84% nucleotide sequence identity with its murine ortholog. The human *POLL* gene encodes a protein of 575 amino acids with an overall 83% amino acid identity to its murine ortholog. Fig. 1 shows a multiple alignment of human pol λ with its mouse ortholog and human pol β. This alignment predicts several structural domains in pol λ: an N-terminal BRCT domain (*light gray* area) and a connecting serine/proline-rich domain, both absent in pol β, and a pol β-like core containing a dRP lyase (8 kDa; *gray* area) and a polymerization (31 kDa; *dark gray* area) domain. Interestingly, the similarity between human and murine pol λ is maximal (92% amino acid identity) in the pol β core (residues 239–573 of murine pol λ and residues 241–575 of human pol λ), very high (84% amino acid identity) in the BRCT domain (residues 35–125 of murine pol λ and residues 36–126 of human pol λ), and lower (55% amino acid identity) in the serine/proline-rich domain (residues 126–238 of murine pol λ and residues 127–240 of human pol λ). Despite an overall lesser similarity, the conservation in this domain appears to be more restricted to serine, threonine, and proline residues, thus emphasizing the putative role of this region as a target for phosphorylation (20). The amino acid similarity among human pol λ and human pol β (about 33% amino acid identity) was high enough to build a computer-generated structural model of the pol β core of human pol λ, whose architecture and subdomain composition

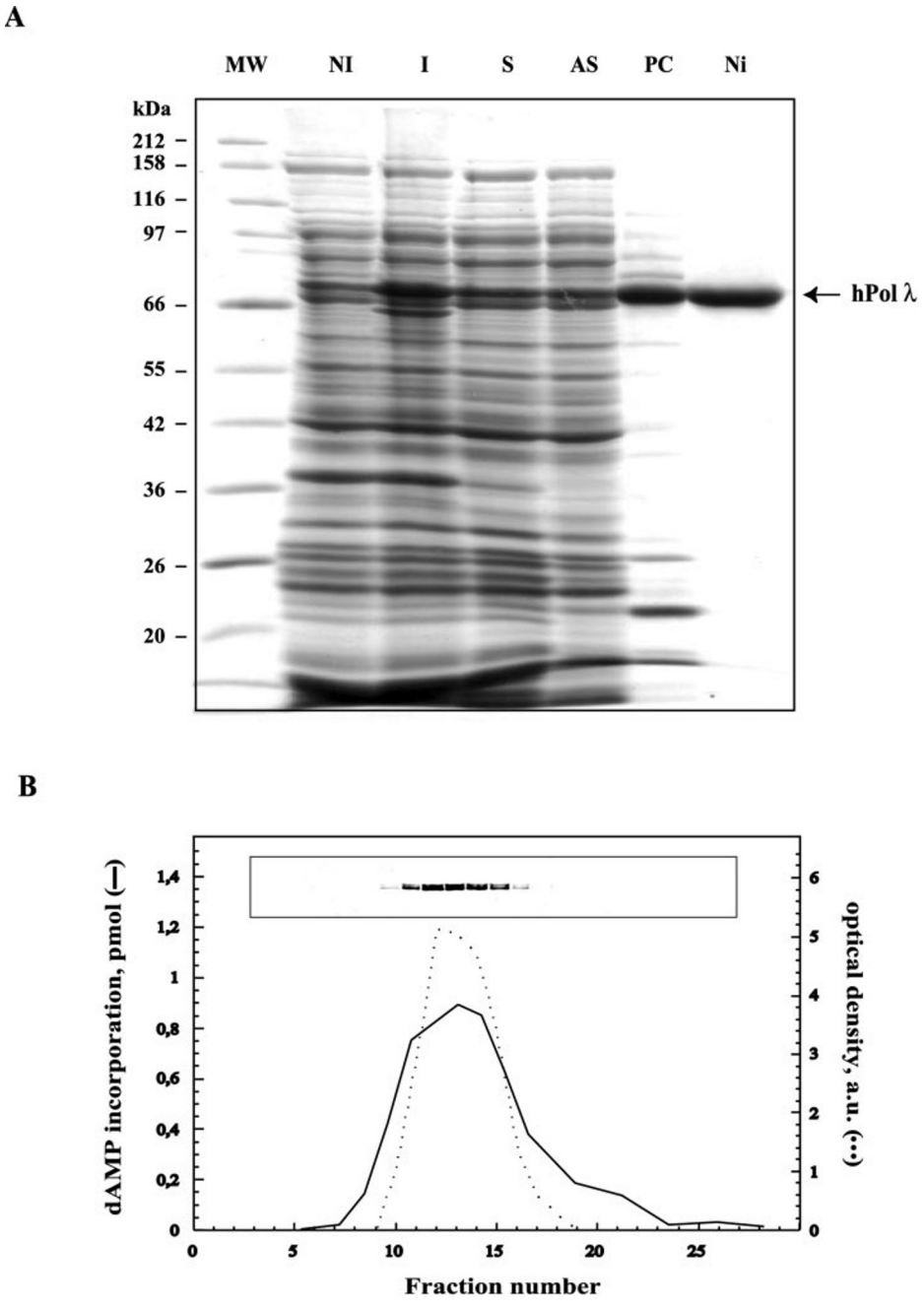

FIG. 2. **Purification of human pol λ.** *A*, overexpression and purification of human pol λ in *E. coli.* Coomassie Blue staining after SDS-PAGE separation of control noninduced (*NI*) and IPTG-induced (*I*) total extracts obtained from *E. coli* cells transformed with the recombinant plasmid pRSET-hpolλ and further purification steps of the IPTG-induced extract (described in detail under "Experimental Procedures") are shown. The electrophoretic mobility of the overproduced pol λ, present in the IPTG-induced total extract, was compatible with its deduced molecular mass (68 kDa/604 amino acids). After cell lysis, an important fraction of pol λ remained soluble (*S*). After polyethyleneimine precipitation of the DNA, pol λ was precipitated with ammonium sulfate at 65% saturation (*AS*), and further purified by PC and Ni-NTA (*Ni*) chromatography, as described under "Experimental Procedures." The electrophoretic migration of a collection of molecular mass markers (*MW*) is shown on the *left*. *B*, cosedimentation of a DNA polymerase activity with the human pol λ 68-kDa polypeptide. Purified human pol λ was subjected to a glycerol gradient sedimentation (see "Experimental Procedures"). The *inset* shows the analysis of fractions 4–26 in a Coomassie Blue-stained SDS-polyacrylamide gel. DNA polymerase activity of each fraction was assayed on activated DNA and is expressed as dAMP incorporation (in pmol). Quantitation of the human pol λ protein band was carried out by densitometry of the stained gel and is expressed as optical density (*a.u.*, arbitrary units).

(fingers, palm, thumb, and 8 kDa) are fully compatible with the three-dimensional structure of pol β (not shown). A closer inspection of the predicted C-terminal pol β core indicates that most residues characteristic of pol X family enzymes, including critical residues involved in dNTP binding, metal binding, DNA binding, and catalysis in pol β, are also invariant or highly conserved in human pol λ.

Analysis of pol λ mRNA Expression—In agreement with the meiotic function proposed for pol λ (20), Northern blot analysis of human pol λ mRNA reflected high expression levels in testis (not shown and Ref. 22). However, a more sensitive reverse transcription-PCR technique detected pol λ mRNA in every human and murine tissue examined (not shown). We therefore performed a quantitative PCR analysis of pol λ expression in human tissues comparing its expression levels with those of pol β, an enzyme thought to have a general role in DNA repair partly because of its housekeeping expression. Relative expression levels of both pol λ and pol β mRNA varied in the different tissues examined, particularly in testis and brain, where pol λ mRNA levels were clearly higher relative to those of pol β (2- and 4-fold, respectively). An opposite situation occurs in liver, where the lowest pol λ:pol β ratio (1:10) was observed. Because pol λ expression is not only restricted to germinal cells, an additional role(s) of this enzyme in somatic cells must be considered. The observation that the relative expression of pol λ and pol β varies in some tissues suggests that the cellular functions of these two enzymes may be nonredundant.

pol λ Has an Intrinsic DNA Polymerase Activity—Human pol λ was expressed in *E. coli* cells as a fusion protein containing a histidine tag at its N terminus (see "Experimental Procedures"). As shown in Fig. 2, upon IPTG induction, a new polypeptide was observed migrating at the expected position for pol λ (~68 kDa) and making up to 1% of the total protein extract. About 25% of the overproduced pol λ remained soluble and was precipitated with 65% ammonium sulfate. Polymerase λ was further purified using PC and Ni-NTA chromatography.

TABLE I
DNA polymerization on activated DNA and poly(dA) · oligo(dT)

DNA polymerization activity was assayed in the standard conditions described under "Experimental Procedures." Individual components were added or omitted as indicated. The ratio of ddTTP to dTTP is indicated in parentheses. 100% activity values correspond to 30 (activated DNA) and 8.5 poly(dA · dT) fmol min^{-1} pmol of $enzyme^{-1}$ for pol λ and to 3 (activated DNA) and 7 poly(dA · dT) fmol min^{-1} pmol of $enzyme^{-1}$ for pol β.

Reaction components (added or omitted)	DNA polymerase activity	
	pol λ	pol β
	%	%
Activated DNA		
None	100	100
−DNA	<1	<1
$-MgCl_2$	<1	<1
$-MgCl_2$ + $MnCl_2$ (1 mM)	134	145
+ ddTTP (1:1)	89	98
+ ddTTP (10:1)	40	69
+ ddTTP (100:1)	3	25
Poly(dA) · oligo(dT)		
None	100	100
−DNA	<1	<1
$-MgCl_2$	<1	<1
$-MgCl_2$ + $MnCl_2$ (1 mM)	1960	3290
+ ddTTP (1:1)	42	98
+ ddTTP (10:1)	38	85
+ ddTTP (100:1)	2	75

The protein eluted from the Ni-NTA column as a homogeneous species as judged by SDS-PAGE and was devoid of nuclease contaminants, as tested in nuclease assays.

To analyze DNA polymerization activity in the purified pol λ fraction, we carried out different *in vitro* assays using either activated DNA or defined homopolymeric molecules as a substrate. As summarized in Table I, the purified pol λ fraction was able to catalyze dNMP incorporation in the presence of either Mg^{2+} or Mn^{2+} divalent metal ions.

As a control of specificity, we carried out a parallel purification from control *E. coli* cells (transformed with the pRSET plasmid). In this case, no polymerization activity was detectable in the final fractions (not shown). To ascertain further that the DNA polymerase activity present in the final purification fraction was intrinsic to pol λ, we demonstrated the cosedimentation in a glycerol gradient of the DNA polymerase activity with the 68 kDa pol λ protein peak, identified by Coomassie Blue staining after SDS-PAGE analysis of each gradient fraction (Fig. 2*B*).

In our standard assay, pol λ specific activity (30 fmol·min^{-1}·pmol of $enzyme^{-1}$) was 10 times higher compared with that of pol β (3 fmol·min^{-1}·pmol of $enzyme^{-1}$) (Table I). Interestingly, replacing magnesium ions with manganese when using poly(dA)·oligo(dT) as a substrate stimulated pol λ and pol β polymerization up to 20-fold and 30-fold, respectively. This substrate-specific effect could be explained if manganese ions facilitate the slippage capacity of both enzymes. As happens with pol β and other pol β-like enzymes (15), ddNTPs inhibited polymerization by pol λ. The inhibition observed for pol λ is in agreement with the weak discrimination for the 3′-OH group of the incoming nucleotide displayed by family X DNA polymerases.

pol λ Lacks 3′ → 5′ Exonuclease—The 3′ → 5′ exonuclease active site of all proofreading eukaryotic and prokaryotic DNA polymerases is made up of three conserved amino acid motifs, named Exo I, Exo II, and Exo III (25). Similar motifs could not be identified in pol λ sequence, suggesting that, as other family X enzymes, pol λ has no proofreading activity. This prediction was confirmed by exonuclease assays (see "Experimental Procedures"), where purified pol λ failed to display any nucleolytic activity both on single stranded and template-primer substrates: a 30-fold excess of enzyme over substrate did not produce a detectable degradation (less than 0.1%) after 30 min at 37 °C (not shown).

pol λ Is Template-dependent and Distributive on a Template-Primer—Although pol β is the closest homolog of pol λ, the latter enzyme also shares significant sequence similarity with both terminal deoxynucleotidyl transferase and pol μ, two enzymes reported to display a template-independent DNA polymerization capacity. To assay whether pol λ is template-dependent or not, DNA molecules of defined sequence were used as substrates to assay DNA polymerization. Whereas pol λ was able to perform synthesis on a template-primer, it was unable to use single stranded DNA or blunt double stranded DNA (not shown), thus suggesting that pol λ is strictly a template-dependent enzyme.

Processivity is a common feature of DNA polymerases involved in extensive DNA synthesis (*i.e.* replicative polymerases), as a direct consequence of tight DNA binding and efficient nucleotide insertion. Conversely, DNA repair enzymes frequently display weaker DNA interactions and incorporate nucleotides more slowly and consequently synthesize DNA in a distributive mode. We used a template-primer and different enzyme:DNA ratios to study the processivity of human pol λ. On this template, pol λ was absolutely distributive both in the presence of Mg^{2+} and Mn^{2+} activating ions, because the length of the synthesized products was strongly dependent on the enzyme:DNA ratio (not shown). Although it can not be discarded that accessory proteins could modulate processivity, these results suggest that pol λ is not well suited to carry out long patch DNA synthesis *in vivo*.

Processive Gap Filling by Human pol λ—Although being a distributive polymerase on a template-primer substrate, pol β is known to conduct processive DNA synthesis in small (<6) DNA gaps, believed to be its physiological substrate. Structural evidence (26) suggests that this is the consequence of the additional contacts that are established in a gapped substrate between the N-terminal 8-kDa domain of the enzyme and the DNA downstream to the gap. Among them, the capacity of the 8-kDa domain to bind a terminal 5′-phosphate group is particularly important for both processivity and binding of pol β during gap filling synthesis (8). We therefore compared the processivity of pols λ and β using a template-primer and a 5-nucleotide gap with or without a phosphate group at its 5′-side (Fig. 3*A*). As can be seen in Fig. 3*B*, the processivity of both enzymes was increased in the presence of a phosphate group at the 5′- side of the gap (*right panels*). For pol λ, this increase was strictly dependent on the presence of a phosphate group, whereas pol β showed some increase in processivity (visible at longer times) even in its absence (*middle panels*). However, this phosphate-independent increase in processivity was only seen when the gap had been partially filled (by insertion of 3 nucleotides), suggesting the establishment of specific contacts between pol β and the DNA requiring a defined gap size (1–2 nucleotides). Whereas pol β displayed a significant strand displacement capacity and efficiently inserted one additional nucleotide after filling the gap, pol λ limited its synthesis to the 5 nucleotides of the gap. This imprecise gap filling by pol β, which has been already described (8), can be overcome *in vitro* by association with XRCC1 protein (27). Because pol λ appears to be intrinsically able to restrict its synthesis to the length of the gap, it would be a suitable candidate to participate in a XRCC1-independent gap filling synthesis (28).

Strand Displacement Coupled with Gap Filling Synthesis Is Enhanced by Mn^{2+} Ions—The use of Mn^{2+} ions as metal activators is known to affect both catalytic efficiency and fidelity of several DNA polymerases *in vitro* (29), including family X DNA

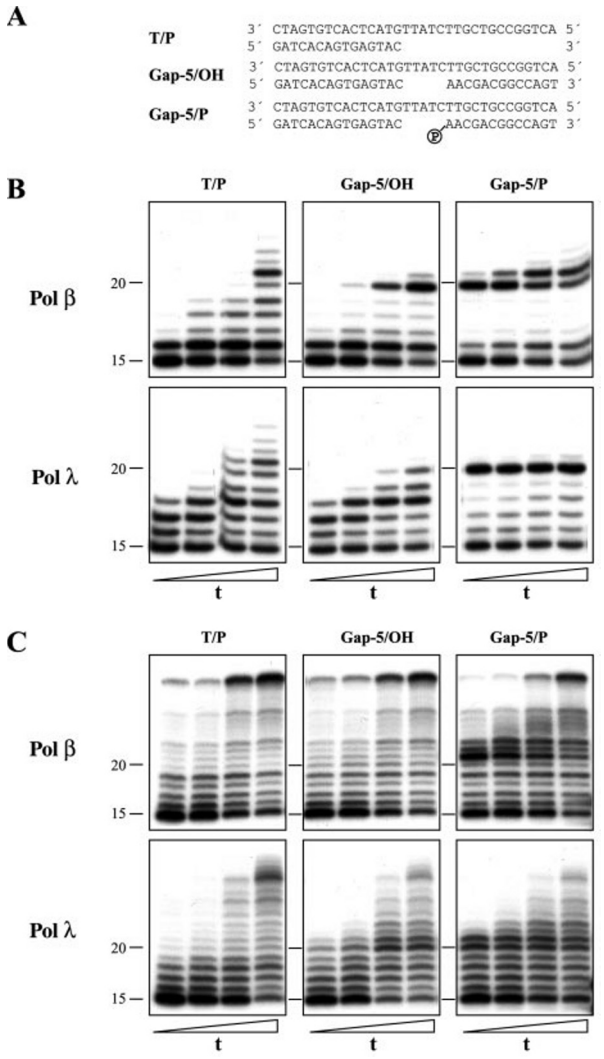

FIG. 3. **Gap filling by pol λ.** *A*, the different molecules used in the analysis were: *T/P*, template-primer; *Gap-5/OH*, 5-nucleotide gap; *Gap-5/P*, 5-nucleotide gap with a 5′-phosphate. Reactions were performed as described under "Experimental Procedures" in the presence of 10 mM $MgCl_2$ (*B*) or 1 mM $MnCl_2$ (*C*), using the substrates indicated and 50 nM pol β or pol λ (as indicated). After incubation for 2, 5, 15, and 30 min at 37 °C, samples were analyzed by 8 M urea and 20% PAGE and autoradiography.

TABLE II
Kinetic constants of dAMP and ddAMP insertion
Kinetic assays were performed as described under "Experimental Procedures."

Steady state				
DNA polymerase	Substrate	V_{max}	$K_{m(app)}$	V_{max}/K_m
		fmol min^{-1}	μM	
pol λ	dATP	0.60 ± 0.02	0.50 ± 0.07	1.2
pol β	dATP	0.46 ± 0.02	7.60 ± 0.70	0.06
Single turnover				
DNA polymerase	Substrate	k_{pol}	K_d	Specificity
		min^{-1}	μM	(k_{pol}/K_d)
pol λ	dATP	0.052 ± 0.001	0.145 ± 0.010	0.358
	ddATP	0.056 ± 0.002	0.104 ± 0.025	0.538
pol β	dATP	0.273 ± 0.007	5.384 ± 0.500	0.050
	ddATP	0.190 ± 0.003	3.358 ± 0.191	0.056

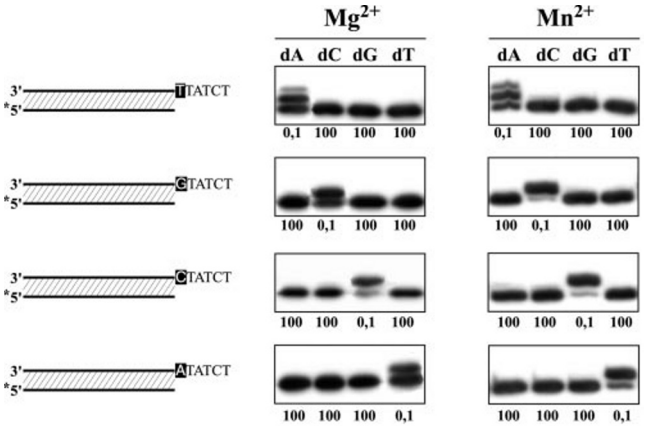

FIG. 4. **Human pol λ preferentially incorporates complementary nucleotides.** Four different template-primer structures were used, differing in the first templating base. Reactions were carried out as described under "Experimental Procedures" using 50 nM pol λ and 10 mM $MgCl_2$ or 1 mM $MnCl_2$ as a source of activating metal ions. Extension of the labeled (*) primer strand in the presence of either the correct (0.1 μM) or the incorrect (100 μM) dNTP was analyzed by 8 M urea and 20% PAGE and autoradiography.

polymerases such as pol μ (19) or pol β (30). Surprisingly, as shown in Fig. 3*C*, manganese greatly stimulated strand displacement synthesis on gapped DNA, catalyzed both by pol β and pol λ. For both enzymes, the extension products obtained on the gapped substrate were comparable with those obtained in the control (no gap) template-primer molecule, and only a slight increase in the proportion of +5 and +6 products revealed that the reaction was occurring in a gapped molecule. Interestingly, this stimulation of strand displacement-coupled DNA polymerization was observed in both the presence or absence of a phosphate group at the 5′-side of the gap, although the presence of this phosphate group slightly constrained the strand displacement capacity of both enzymes.

pol λ Has a High Affinity for dNTPs—An early observation was that the rate of nucleotide incorporation by pol λ on activated DNA was not augmented by using dNTP concentrations over 1 μM. Contrarily, a reduction in nucleotide incorporation was observed when higher amounts (100 μM) of dNTPs were used (data not shown). These data suggested that pol λ was saturated at a low nucleotide concentration. Therefore, a steady-state kinetic analysis of dNMP incorporation on activated DNA was performed in parallel for both pol β and pol λ. The data obtained were fit to the Michaelis-Menten equation (Equation 1) and used to determine apparent K_m values. Interestingly, as shown in Table II, pol λ displayed an $K_{m(app)}$ 15-fold lower than that of pol β (0.50 ± 0.07 and 7.60 ± 0.70 μM, respectively). This difference was investigated further using defined DNA molecules and single turnover conditions, where the enzyme concentration is higher than the concentration of DNA. Under these conditions, DNA binding differences between the two enzymes are minimized, and the kinetic constants reflect the equilibrium binding constant (K_d) and the intrinsic rate of insertion (k_{pol}). For each dNTP concentration, the amount of product formed with time was fit to a single exponential (Equation 2) to derive the observed polymerization rate (k_{obs}). The exponential rate constants (k_{obs}) were then plotted as a function of substrate concentration and fit to a hyperbola (Equation 3) to obtain K_d and k_{pol}. Single turnover analysis of dAMP incorporation clearly revealed that pol λ has a higher affinity for dAMP than pol β. As shown in Table II, a 37-fold difference in the K_d was observed (0.145 ± 0.010 μM for pol λ and 5.384 ± 0.500 μM for pol β).

To analyze 3′-OH discrimination by pol λ further, we used a defined template-primer DNA molecule to compare dAMP and ddAMP incorporation. Polymerase λ was able to insert ddAMP efficiently. The same behavior was observed for pol β, in agree-

ment with previous reports (15, 31). Single turnover parameters (Table II) reflected that dAMP and ddAMP are inserted by both enzymes with a similar efficiency, suggesting that these proteins do not show a strong selection for the 3′-OH group of the nucleotide.

Fidelity of pol λ—We have shown previously that pol λ is a DNA-dependent DNA polymerase with no proofreading activity. As a first analysis of the capacity of pol λ to catalyze faithful DNA synthesis, each of the four dNTPs was assayed individually as a substrate to be incorporated opposite the four possible templating bases, in the presence of Mg^{2+} or Mn^{2+} ions. Fig. 4 shows that in all cases, pol λ preferentially inserted the correct dNTP. Thus, pol λ performs DNA synthesis following the Watson-Crick base pairing rules. For a more quantitative analysis, we then assayed the fidelity of pol λ synthesis while filling a 5-nucleotide gap in a M13mp2 DNA. This DNA substrate produces a colorless M13 plaque phenotype because of a TGA stop codon in the *lacZ* α complementation gene sequence within the gap. As described previously (32) base substitution errors that revert the nonsense codon are scored as blue plaque revertants among total copied and ligated products. Synthesis by pol λ to fill in the gap generated products with a *lacZ* reversion frequency of 9×10^{-4}, which is similar to the reversion frequency observed with pol β. Thus, although pol λ base substitution fidelity is lower than that of replicative polymerases, it is relatively high when compared with that of the polymerases in the recently identified pol Y family (33). DNA sequence analysis of pol λ-generated revertants revealed significant differences in the base substitution specificity of the two enzymes. Polymerase λ predominantly generates transition errors. Note that of the 55 pol λ-produced base substitutions (Table III) only 3 were transversion errors. In contrast pol β-generated base substitutions are more divided evenly between transitions and transversions.

TABLE III
Base substitution specificity of pol λ in short gap reversion assay

The error rates (E.R.) were calculated by dividing the total number of errors in a class by the total number of mutants sequenced (80), then multiplying by the mutant frequency, and then dividing by the target size. This number was then divided by 0.6, the probability of expressing an error in *E. coli*.

Template nucleotide and mispair[a]	pol λ		pol β[b] E.R. × 10^{-4}
	No. of Mutants	E.R. × 10^{-4}	
T			
T · dGMP	24	4.5	29
T · dCMP	0	≤0.19	1.8
T · dTMP	0	≤0.19	≤0.9
G			
G · dAMP	0	≤0.19	1.8
G · dGMP	1	0.19	1.8
A			
A · dCMP	28	5.3	2.7
A · dGMP	1	0.19	6.2
A · dAMP	1	0.19	0.9

[a] Mispair is template/dNTP.
[b] Data are from Ref. 32. The pol β reversion frequency is 26×10^{-4}.

DISCUSSION

Polymerase λ is a recently discovered enzyme belonging to the family X of DNA polymerases (20–22). Amino acid sequence comparison among all members of this family reveals a common domain organization, which could imply a similar catalytic mechanism (34). However, despite these general similarities, family X includes heterodox polymerases such as terminal deoxynucleotidyl transferase, able to conduct template-independent synthesis, and pol μ, also endowed with some degree of template independence and extremely unfaithful (19). Although pol λ is the closest relative of pol β (33% amino acid identity), no evidence had been published to date regarding pol λ polymerization properties.

Unlike its murine ortholog (20), human pol λ could be expressed in *E. coli* in a soluble and active form and purified to homogeneity. Biochemical analysis of human pol λ showed basic features very similar to pol β: 1) strict template dependence when using Mg^{2+} as metal activator; 2) lack of an intrinsic 3′ → 5′ exonuclease; 3) distributive on a template-primer but processive in short gaps having a phosphate group at its 5′-side; 4) low discrimination against ddNTPs; 5) preferential insertion of complementary dNMPs and similar base substitution fidelity. Moreover, as has been shown recently, pol λ has an intrinsic dRP lyase activity (23), an activity that is crucial for the base excision repair pathway. All of these similarities suggest that pol λ is a β-like enzyme that could play a role in DNA repair *in vivo*.

The use of Mn^{2+} ions to activate pol β results in an increased reactivity at the catalytic site, lowering the fidelity of synthesis and even allowing template-independent reactions (30). Here we describe an additional effect of Mn^{2+} ions, increasing the

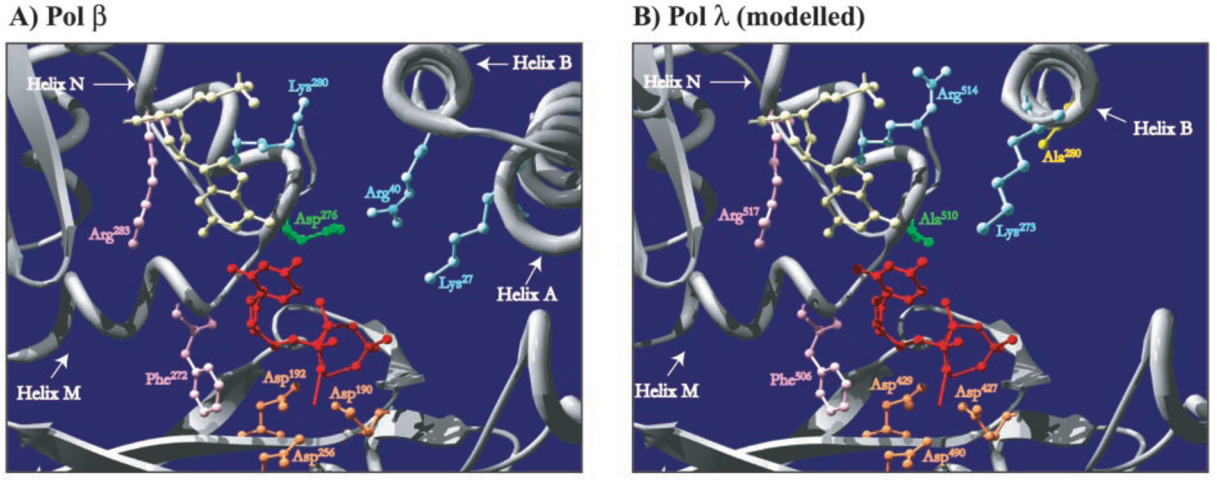

FIG. 5. **Structural basis for nucleotide binding by human pol λ.** The nucleotide binding pocket of human pol β (*A*) is compared with the putative (modeled) nucleotide binding pocket of human pol λ (*B*). The incoming ddCTP (*red*) is shown, hydrogen bonded to its templating base (*light yellow*). Relevant residues are shown (*ball* and *stick*), and their position relative to the N terminus of the protein is indicated. One of the most striking differences between the pol β and pol λ dNTP binding site is the nonconservation of pol β residue Asp^{276} (*green*), which plays a direct role in dNTP binding and selectivity (for details, see "Discussion"). A model structure of the whole β-core of human pol λ, with the exception of α-helix A, was generated with the program Swiss Model (www.expasy.ch/swissmod/swiss-model.html). The figure was made with Swiss PDB Viewer ((42) www.expasy.ch/spdbv/) and rendered with POV Ray (www.povray.org).

strand displacement capacity of both pol β and pol λ. This effect could be the result of Mn^{2+} binding to the helix-hairpin-helix motif of the 8-kDa domain of both polymerases (35), potentially distorting DNA binding and facilitating strand displacement.

Despite all their similarities, pol λ's lack of an apurinic/apyrimidinic lyase activity (23) or its different base substitution specificity compared with pol β reveal that pol λ and pol β are biochemically distinguishable. Moreover, an important difference between these two enzymes is related to the efficiency of dNTP binding and selection. Steady-state kinetic analysis reflected that the $K_{m(app)}$ (dNTP) of pol λ is an order of magnitude lower compared with that of pol β, and consistent with single turnover experiments pol λ has a 37-fold higher affinity for incoming nucleotides than pol β.

Most residues involved in dRP lyase catalysis and 5′-phosphate binding are conserved between pol β and pol λ (23). Moreover, pol λ conserves most pol β key residues related to polymerization catalysis including the three invariant aspartates that coordinate the divalent metal ions (Asp^{427}, Asp^{429}, and Asp^{490}), pivotal residues that ensure fidelity of catalysis by pol β such as Arg^{283} and Phe^{272} (Arg^{517} and Phe^{506} in pol λ, respectively), and other invariant residues in family X polymerases (34). However, in agreement with their biochemical differences, a strong dissimilarity can be observed at the dNTP binding pocket of both enzymes. As illustrated in Fig. 5, pol λ conserves most pol β residues that surround the incoming dNTP in the closed conformation of the ternary complex (26) but differs in some 8-kDa residues presumed to increase dNTP binding in a single nucleotide gap (*i.e.* pol β residues Arg^{40} and Lys^{27} (36)). More interestingly, pol β Asp^{276}, known to make Van der Waals interactions with the base of the incoming nucleotide (11), is replaced by Ala^{510} in pol λ. Besides forming the only electrostatic interaction between α-helix N and the 8-kDa domain of pol β (through hydrogen bonding with Arg^{40} (36)), Asp^{276} is believed to restrict dNTP binding. Indeed, replacing Asp^{276} with an uncharged residue (*i.e.* valine or glycine) results in an apparent increase in the nucleotide binding affinity (37). Moreover, a D276V mutant of pol β has a 4–9-fold increased nucleotide binding affinity compared with that of the wild-type enzyme (36). Accordingly, pol λ, having an uncharged residue at this position (Ala^{510}), has a 37-fold higher nucleotide binding affinity than pol β, thus behaving similarly to the D276V mutant of pol β.

As shown here, it can be predicted that at a low dNTP concentration, pol λ would be a much more active enzyme than pol β. This biochemical difference suggests that both enzymes, instead of having a redundant function, could play a specific role related to the cellular concentration of nucleotide precursors for DNA synthesis. Thus, DNA repair could take advantage of a dual functional solution similar to that described for glucose phosphorylation, which involves both a low K_m hexokinase and a high K_m glucokinase (hexokinase IV (38)). Interestingly, pol λ mRNA expression is apparently cell cycle-dependent, being higher in quiescent and S to M phase cycling cells (22). Thus, pol λ could be specialized in DNA repair taking place during specific stages of the cell cycle, required in non-proliferative cell types or during differentiation processes. Such a specialized DNA repair function in pol λ could be perhaps related to its N-terminal BRCT domain. This domain, absent in pol β but present in a large number of nuclear proteins, has been suggested to take part in different protein-protein and protein-DNA interactions (39–41). Therefore, an important difference with pol β could be the potential to establish (via its BRCT domain) distinct interactions that could regulate pol λ cellular function. Further work should be carried out to ascertain the specific pathway(s) recruiting this enzyme and its contribution to the maintenance of genetic stability.

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