Published online August 24, 2005

4762–4774 *Nucleic Acids Research, 2005, Vol. 33, No. 15*
doi:10.1093/nar/gki780

Characterization of *Sp*Pol4, a unique X-family DNA polymerase in *Schizosaccharomyces pombe*

Sergio González-Barrera, Arancha Sánchez, José F. Ruiz, Raquel Juárez, Angel J. Picher, Gloria Terrados, Paula Andrade and Luis Blanco*

Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Universidad Autónoma, Madrid, Spain

Received May 25, 2005; Revised and Accepted August 3, 2005

ABSTRACT

As predicted by the amino acid sequence, the purified protein coded by *Schizosaccharomyces pombe* SPAC2F7.06c is a DNA polymerase (*Sp*Pol4) whose biochemical properties resemble those of other X family (PolX) members. Thus, this new PolX is template-dependent, polymerizes in a distributive manner, lacks a detectable 3′→5′ proofreading activity and its preferred substrates are small gaps with a 5′-phosphate group. Similarly to Polμ, *Sp*Pol4 can incorporate a ribonucleotide (rNTP) into a primer DNA. However, it is not responsible for the 1–2 rNTPs proposed to be present at the mating-type locus and those necessary for mating-type switching. Unlike Polμ, *Sp*Pol4 lacks terminal deoxynucleotidyltransferase activity and realigns the primer terminus to alternative template bases only under certain sequence contexts and, therefore, it is less error-prone than Polμ. Nonetheless, the biochemical properties of this gap-filling DNA polymerase are suitable for a possible role of *Sp*Pol4 in non-homologous end-joining. Unexpectedly based on sequence analysis, *Sp*Pol4 has deoxyribose phosphate lyase activity like Polβ and Polλ, and unlike Polμ, suggesting also a role of this enzyme in base excision repair. Therefore, *Sp*Pol4 is a unique enzyme whose enzymatic properties are hybrid of those described for mammalian Polβ, Polλ and Polμ.

INTRODUCTION

Efficient DNA repair is essential to maintain genome stability and cell viability (1,2). In spite of a variety of DNA repair mechanisms, one common step is DNA synthesis, carried out by specialized DNA polymerases. DNA polymerases are classified into four different groups according to their biochemical properties and to the biological processes in which they are involved. Among them, only family X DNA polymerases (PolX) are devoted to DNA repair, being evolutionarily conserved in prokaryotes, eukaryotes and archaea (3–5). However, their number ranges from five members in mammals [Polβ, Polλ, Polμ, terminal deoxynucleotidyltransferase (TdT) and Polσ] to one member in yeasts, plants, and some bacteria and viruses, i.e. *Saccharomyces cerevisiae* (*Sc*Pol4), *Arabidopsis thaliana* (*Ath*PolX), *Bacillus subtilis* (*Bs*PolX) and *African swine fever virus* (*ASFV*PolX). Interestingly, two model organisms, *Caenorhabditis elegans* and *Drosophila melanogaster*, whose genomes have been completely sequenced, have no putative PolX (3).

PolX enzymes most probably share a common modular organization (Polβ core) consisting of an 8 kDa domain and a 31 kDa polymerization domain comprising 'fingers', 'palm' and 'thumb' subdomains. Such a structural organization has been demonstrated for Polβ (6,7), TdT (8), Polλ (9,10) and *ASFV*PolX (11,12). Unlike Polβ, *ASFV*PolX, bacterial and archaea PolX members, other family enzymes (Polλ, Polμ, TdT and *Sc*Pol4) have an additional domain, the Brca1 C-terminal, named BRCT, which has been suggested to take part in protein–protein and protein–DNA interactions (13). Besides this BRCT domain, Polλ *Ath*PolX and *Sc*Pol4 have a proline/serine-rich region in their central part with a yet unknown function (3).

Regarding their biochemical properties, all DNA polymerases from this family are single-subunit enzymes, lacking the 3′→5′ exonuclease activity and displaying very low processivity during primer extension reactions [reviewed in (14)]. Polβ, the paradigm of the PolX family, inserts nucleotides in a template-dependent manner and is moderately accurate (15,16). Its preference for small gaps with a 5′-phosphate group (17) and its deoxyribose phosphate (dRP) lyase activity that relies on the 8 kDa domain (18) are properties consistent with a role in base excision repair (BER), a major pathway

*To whom correspondence should be addressed. Tel: +34 91 497 8493; Fax: +34 91 497 4799; Email: lblanco@cbm.uam.es
Present address:
José F. Ruiz, Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Spain

involved in the repair of damaged nucleotides (19,20). This multistep process is initiated with the removal of the modified base by a specific DNA *N*-glycosylase yielding apurinic/apyrimidinic (AP) sites. AP sites are recognized and incised by an AP endonuclease leaving single strand breaks (SSBs) with a 5′-dRP end. Finally, the 5′-dRP can be released either by the 5′-dRPase activity of Polβ (short-patch BER) or by the combined action of a DNA polymerase (Polβ, and Polε or Polδ) and the 5′-flap endonuclease FEN1 (long-patch BER) (21,22).

Polλ has 32% amino acid identity to Polβ and contains an intrinsic dRP lyase activity that can substitute for Polβ in BER *in vivo* and *in vitro* (23,24). However, the high affinity of Polλ for deoxynucleotides (dNTPs) (37-fold over Polβ) is consistent with its possible involvement in DNA transactions occurring under low cellular levels of dNTPs, i.e. in non-replicating phases of the cell cycle (25). Similar to Polβ, Polλ inserts dNTPs in a DNA template-dependent manner and is processive in small gaps containing a 5′-phosphate group (25). In addition, immunodepletion of nuclear extracts of HeLa cells (26) and recent studies in which Polλ associates with a Ku-XRCC4–DNA ligase IV–DNA complex (27–29) suggest a possible role for Polλ in non-homologous end-joining (NHEJ).

Polμ has 41% identity to TdT, a template-independent DNA Pol X responsible for the N-addition during V(D)J recombination of the immunoglobulin genes and T-cell receptor genes (30,31). Polμ-deficient mice are impaired in V(D)J recombination of the immunoglobulin κ light chain (32), which is initiated by an induced double strand break (DSB) that is repaired by an NHEJ mechanism, similar to that employed by other tissues to repair DSBs. However, unlike TdT, whose expression pattern is restricted to lymphoid tissues, Polμ is expressed in additional tissues (33), suggesting a more general role of Polμ in DNA repair (34).

Polμ behaves as an error-prone DNA polymerase, since it is able to induce/accept dislocations of the template strand (35). Unlike Polβ and Polλ, Polμ is able to insert ribonucleotides (rNTPs) to a DNA chain (36,37) and lacks dRP lyase activity (24). Based on these properties and on the physical and functional interactions with the Ku-XRCC4–DNA ligase IV–DNA complex (38), it has been proposed that Polμ functions in NHEJ and V(D)J recombination by promoting microhomology search and pairing activities (27,29). Polμ is not a strictly template-dependent DNA polymerase, since it has an intrinsic terminal transferase activity (33) that probably plays a role in microhomology-mediated NHEJ reactions (R. Juárez, J. F. Ruiz, S. A. Nick McElhinny, D. A. Ramsden and L. Blanco, manuscript submitted).

In contrast to mammals, budding and fission yeasts have only one DNA PolX enzyme (3). Whereas *Sc*Pol4 is closely related to Polλ (25% amino acid identity to the Polλ core), the putative DNA PolX from the fission yeast *Schizosaccharomyces pombe* (SPAC2F7.06c) is more closely related to Polμ than to Polλ (27% versus 24% identical core residues, respectively). *Sc*Pol4 was the first DNA PolX shown to play a role in NHEJ (39). In agreement with that, it has been shown to have a direct interaction of the BRCT domain of *Sc*Pol4 with the Lig4/Lif1 complex (40), and a physical and functional interaction of Rad27 with both *Sc*Pol4 and Dnl4/Lif1 (41). No functional data have been reported for the putative DNA PolX (SPAC2F7.06c) from the fission yeast *S.pombe*, a unicellular eukaryotic organism whose properties closely resemble those of higher eukaryotic organisms. For this reason, *S.pombe* is a good model system for the analysis of gene products involved in DNA repair. Here, we report the cloning, expression and biochemical characterization of the SPAC2F7.06c gene product from *S.pombe*. DNA polymerization properties and the presence of a dRP lyase activity support a role of this DNA polymerase in both NHEJ and BER reactions. In spite of the closer similarity to Polμ, this enzyme combines Polβ, Polμ and Polλ properties, and therefore, it should be more unambiguously referred to as *Sp*Pol4.

MATERIALS AND METHODS

Strains and growth conditions

Cells were grown at 30°C in rich medium (YES; 0.5% yeast extract, 3% glucose and supplemented with 200 mg/l of leucine and uracil) or in minimal medium (EMM). Appropriate amino acids and thiamine were added to EMM when required to a final concentration of 200 mg/l and 25 μM, respectively. Geneticin selection was performed using YES medium containing 100 mg/l G418 (Sigma). The *pol4*Δ::KanMX strains, sp8 and sp10, were created from the wild-type strains, sp7 (*h- leu1-32 ura4D18*) and sp968 (h^{90}), respectively, using the PCR-based method and the primers, pol4.A (5′-TCCCTTAGTTGTAATTGTTCAAAATGAAG-ATTCTTGCAAG**CAGCTGAAGCTTCGTACGCT-**3′) and pol4.B (5′-AGTAATGTGGCGATCTTAAGGTCAAGATA-G-GTATTTACTACTAGTGGATCTGATATCATC-3′). The nucleotide sequences in boldface overlap to the KanMX cassette of plasmid pFA6a-kanMX4 (42). The deletion was confirmed by PCR using primers pol4.C (5′-AGATCTGTT-CAAAATGAAGATT-CTTGC-3′) and pol4.D (5′-CTGCA-GAGTAATGTGGCGATCTTA-AGG-3′) and by Southern blot (data not shown).

Nucleotides and proteins

Ultrapure unlabeled dNTPs and rNTPs, [γ-^{32}P]ATP, [α-^{32}P]dCTP and [α-^{32}P]ddATP (3000 Ci/mmol) were purchased from Amersham Biosciences. T4 polynucleotide kinase, UDG and T4 DNA ligase were purchased from New England Biolabs; TdT was obtained from Promega; restriction endonucleases and *Taq* Expand High Fidelity were obtained from Roche; hAPE was a gift from Dr S. H. Wilson (NIEHS, Research Triangle Park, NC). Purified human Polλ and Polμ were obtained as described previously (25,33).

Oligonucleotides, templates and substrates for DNA polymerization

Synthetic DNA oligonucleotides were obtained from Invitrogen [P15, 5′-TCTGTGCAGGTTCTT-3′; P15 (C), 5′-TCTGTGCAGGTTCTC-3′; SP1C, 5′-GATCACAGTGA-GTAC-3′; P6, 5′-CTGCAGCTGATGCGCUGTACGGATC-CCCGG-GTAC-3′; T32 (A), 5′-TGAAGTCCCTCTCGAC-AAAGAACCTGCACAGA-3′; T32 (C), 5′-TGAAGTCCCT-CTCGACCAAGAACCTGCACAGA-3′; T32 (G), 5′-TGAA-G-TCCCTCTCGACGAAGAACCTGCACAGA-3′; T32 (T),

5′-TGAAGTCCCTCTCGACTAAGAACCTGCACAGA-3′; T13 (C), 5′-AGAAGTGTATCTCGTACTCACTGTGATC-3′; T18 (T), 5′-ACTGGCCGTCGTTCTATTGTACTCACTGTGATC-3′; T4, 5′-GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG-3′; DG1, 5′-AGATACACTTCT-3′; DG5, 5′-AACGACGGCCAGT-3′; D16, 5′-GTCGAGAGGGACTTCA-3′; D15, 5′-TCGAGAGGGACTTCA-3′]. All the oligonucleotides were purified by 8 M urea–20% PAGE. Oligonucleotides SP1C, P15 (C), P15, P6 and oligo(dT)$_{15}$ were 5′-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. For dRP lyase activity assay, oligonucleotide P6 was 3′-labeled with [α-^{32}P]ddATP and TdT. Polymerase activity was evaluated by using synthetic double-stranded oligonucleotides as substrates. These substrates were prepared by annealing a 5′-^{32}P-end-labeled primer to different oligonucleotides to generate open (P15/T32 or SP1C/T18(T) and 1 or 2 nt gapped (P15/T32/D16; SP1C/T13(C)/DG1 or P15/T32/D15) template/primer substrates in the presence of 0.2 M NaCl and 60 mM Tris–HCl, pH 7.5. The polymerization reactions were done in 12.5 µl of incubation mixture containing 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$ or 1 mM $MnCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, different concentrations of the indicated dNTPs or rNTPs, 4 nM of 5′-labeled substrate and the indicated concentrations of purified hPolµ, hPolλ, *Sp*Pol4 or calf thymus TdT. After incubation for 15 min at 30°C, reactions were stopped by adding gel loading buffer [95% (v/v) formamide, 10 mM EDTA, pH 8, 0.1% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue]. Products were resolved and analyzed by denaturing 8 M urea–20% PAGE and autoradiography. Quantification was done in a Fujix BAS1000.

Cloning and purification of *S.pombe* DNA polymerase X

Cloning of the *S.pombe Sp*Pol4 gene was started from the identification of an open reading frame (ORF) (SPAC2F7.06c), in the public database *S.pombe*/GeneDB (http://www.genedb.org/) that codifies for a putative DNA polymerase from the X family. Specific primers with restriction sites (in boldface) in their 5′ ends *Sp*Pol4.5′BgX (5′-**AGATCTTGCTCGAGC**ATGAAGATTCTTGCAAGAAGATTCTTGCAAGCA-3′) and *Sp*Pol4.3′STOPNBg (5′-**AGATCTGCGGCCGCC**CTATCCCGTGTTACGAAACTTT) were designed to amplify yeast genomic DNA. PCR was performed with *Taq* Expand High Fidelity (Roche) as follows: 35 cycles at 95°C for 30s, 50°C for 30s and 68°C for 120s. The 1551 bp *Sp*Pol4 PCR product was cloned in pGEM-T Easy (Promega) to generate plasmid pGEM-T Easy::*Sp*Pol4, verified by sequencing, digested with BglII and subcloned in the BamHI site of the expression vector pDS473a, which allows the expression of recombinant proteins as fusions with a glutathione *S*-transferase (GST)-tag, to generate the yeast expression plasmid pDS473-*Sp*Pol4. Expression of *Sp*Pol4 was carried out in the *S.pombe* wild-type strain sp7 transformed with plasmid pDS473-*Sp*Pol4. A 10 liters culture was grown at 30°C for 18–20 h in EMM supplemented with leucine (final $OD_{600} = 1$). Subsequently, the cultured cells were harvested at 4°C and washed with stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA and 1 mM NaN_3, pH 8). The pelleted cells were weighted (10 g) and frozen (−70°C). Just before purification, which was carried out at 4°C, frozen cells were thawed on ice in the presence of 50 ml ice-cold lysis buffer [phosphate-buffered saline (PBS), 50 mM NaF, 2 mM EDTA, pH 8, 1% NP-40, 1.3 mM *p*-NH_2-benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 tablet of protease inhibitor cocktail; Roche] and broken with a French Press (twice at 20 000 psi). KCl was added to the lysate upto a final concentration of 0.2 M. Cell debris were separated from the soluble lysates by ultracentrifugation (50 000 *g* for 1 h at 4°C in a Beckman JA-25.50 rotor). The protein from the soluble fraction was subjected to affinity chromatography. Gluthathione–Sepharose 4B (1 ml) (Pharmacia) packed into a column was equilibrated with 10 vol of lysis buffer. Soluble lysate was then loaded at 3 ml/h flow rate into the column. Afterwards, the column was extensively washed with buffer IPP150 (PBS and 0.1% NP-40) and equilibrated with native binding buffer (100 mM Tris–HCl, pH 8 and 100 mM NaCl). After several washing steps with native binding buffer containing 20 mM gluthathione, polymerase-containing fractions estimated by Coomassie blue staining were collected, 2-fold diluted with buffer A (50 mM Tris–HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA and 1 mM DTT), and bound to a phosphocellulose column (1 ml) and eluted with buffer A containing 500 mM NaCl. This fraction contains highly purified GST-tagged *Sp*Pol4. Protein concentration was estimated by densitometry of Coomassie blue-stained 10% SDS–PAGE gels, using standards of known concentration. Under these conditions, the yield was 26 µg of purified GST-tagged *Sp*Pol4/g of *S.pombe* cells. This purified final fraction, adjusted to 50% (v/v) glycerol and supplemented with 0.1 mg/ml BSA, was stored at −70°C.

Construction and purification of a polymerization-deficient form of *Sp*Pol4

Site-directed mutations were introduced into pGEM-T Easy::*Sp*Pol4 plasmid by using a PCR-based method (QuickChange® Site-Directed Mutagenesis kit; Stratagene) with the oligonucleotide 5′-GCCTGTTGGAGCGGCCGTTGCTATGGTGTTGAGTCC-3′ and its reverse complementary oligonucleotide 5′-GGACTCAACACCATAGCAACGGCCGCTCCAACAGGC-3′ for the double mutation D355A/D357A. The plasmid pGEM-T Easy::*Sp*Pol4$^{D355A/D357A}$ generated was sequenced and a BglII fragment containing the *Sp*Pol4$^{D355A/D357A}$ sequence was subcloned in the BamHI site of pDS473a. *Sp*Pol4$^{D355A/D357A}$ protein, which has two of the three catalytic aspartates mutated to alanines, was purified to homogeneity as the wild-type *Sp*Pol4 described above.

DNA polymerization on activated DNA

The incubation mixture contained, in 25 µl, 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 13.2 nM [α-^{32}P]dCTP, 1 µM (dATP, dCTP, dGTP, dTTP), 1 mM $MnCl_2$ or 10 mM $MgCl_2$ as metal activator, 625 ng of activated calf thymus DNA and 250 nM of the purified GST-tagged *Sp*Pol4 or *Sp*Pol4$^{D355A/D357A}$. After incubation for 30 min at 37°C, the reactions were stopped by adding 10 mM TE/0.1% SDS and the samples were filtered through Sephadex G-50 spin columns in 10 mM TE/0.1% SDS. The excluded volume, corresponding to the labeled DNA, was counted (Liquid Scintillation Counter; Pharmacia) and the

polymerization activity of *Sp*Pol4 was calculated as the amount of incorporated dCMP.

3′→5′ exonuclease assay

The incubation mixture, in 12.5 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 *Sp*Pol4 and 1.5 nM single-stranded labeled P15 or P15/T32(C) hybrid. Reactions were incubated at 30°C for 15 min and were stopped by adding denaturing loading buffer. 3′→5′ exonucleolysis, expected to produce a degradation ladder of the labeled P15 primer, was analyzed by 8 M urea–20% PAGE and autoradiography.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using 1 [SP1C/T13(C)/DG1] and 5 nt [SP1C/T18(T)/DG5] gapped molecules to analyze the interaction of *Sp*Pol4 and DNA. Gel mobility shift assays were performed in a final volume of 12.5 µl containing 50 mM Tris–HCl, pH 7.5, 0.1 mg/ml BSA, 1 mM DTT, 4% glycerol, 4 nM labeled DNA and different concentrations of *Sp*Pol4 (250, 600 and 1200 nM). Samples were incubated for 10 min at 30°C to allow the formation of enzyme–DNA complexes. For competition analysis, 100 nM *Sp*Pol4 was incubated with labeled 1 nt gapped 5′-phosphate molecules (4 nM) for 15 min at 30°C in a final volume of 25 µl. After the complexes were formed, unlabeled 1 nt gapped 5′-phosphate (200 and 600 nM) or 1 nt gapped 5′-hydroxyl (200 and 600 nM) molecules were added to the reaction mixture and incubated for another 10 min at 30°C. After incubation, samples were mixed with 3 µl of 30% glycerol and resolved by native gel electrophoresis on a 4% polyacrylamide gel (80:1 monomer/bis). After autoradiography, DNA polymerase–DNA complexes were detected as mobility retardation in the migration position of the labeled free DNA. Quantification of the competition experiments was done in a Fujix BAS1000. The amount of the labeled GAP1-P forming *Sp*Pol4::GAP1-P complexes was calculated by normalizing the radioactive signal in the shifted band to the total radioactivity.

dRP lyase activity assay

As a substrate, the 3′ end 34mer-labeled P6 oligonucleotide was annealed to the 34mer T4 oligonucleotide. This labeled double-stranded substrate (500 nM) was treated with UDG (100 nM) for 20 min at 37°C in buffer containing 50 mM HEPES, pH 7.5, 20 mM KCl and 2 mM DTT to remove the uracil. After incubation, the mixture was supplemented with 10 mM $MgCl_2$ and 40 nM hAPE for 10 min at 37°C; thus, generating the substrate for dRP lyase activity. Reaction mixtures (25 µl) containing 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, 70 nM concentration of the treated substrate and different amounts of either *Sp*Pol4 (20, 60 and 120 nM), *Sp*Pol4$^{D355A/D357A}$ (20, 60 and 120 nM), hPolµ (70 nM) or hPolλ (60 nM) were incubated at 37°C for 20 min. After incubation, $NaBH_4$ was added to a final concentration of 340 mM, and the reactions were kept for 20 min on ice. Stabilized (reduced) DNA products were ethanol precipitated in the presence of 0.1 g/ml of tRNA, resuspended in water and analyzed with 8 M urea–20% PAGE and visualized by autoradiography.

In vitro reconstitution of BER

A 34mer double-stranded DNA substrate that contained a G opposite uracil at position 16 was used. This molecule was treated as described above for the dRP lyase activity assay to generate the dRP-containing substrate. Reactions (25 µl) containing 70 nM substrate, 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, 0.3 µM [α-^{32}P]dCTP and either *Sp*Pol4 (125 nM) or human Polλ (245 nM) were incubated for 20 min at 37°C. Later, each reaction was divided into two halves. One was incubated with 1 mM ATP and 40 U T4 DNA ligase for 10 min at 37°C and the other was mock-treated. Reactions were terminated by the addition of denaturing loading buffer, analyzed by 8 M urea–20% PAGE and visualized by autoradiography.

Genomic DNA preparation and imprint analysis in *S.pombe*

Yeast chromosomal DNA was purified from a logarithmically growing culture (10 ml; OD_{600} ~0.5–1). Cells were harvested and resuspended in 200 µl breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 8 and 1 mM EDTA), ~200 µl glass beads and 200 µl phenol/chloroform/isoamyl alcohol (25:24:1). After 15 min vortexing at high speed, 200 µl TE was added and the mixture was centrifuged for 5 min at 11 000 *g*. The aqueous layer was transferred to a clean tube and DNA was precipitated with ethanol. The pellet was resuspended in TE. HindIII-digested DNA (50 µg) was separated by agarose gel electrophoresis and analyzed by Southern hybridization using a 1 kb ^{32}P-labeled *mat1-P* PCR probe. The oligonucleotides sequences used for the PCR were *mat1*-5′, 5′-AGAAGAGAGAGTAGTTGAAG-3′; and *mat1P*-3′, 5′-CCAATTCCTTCT-TGTATATGTTA-TAC-3′. The *mat2* (6.3 kb) and *mat3* (4.2 kb) bands result from hybridization to the *mat1* probe as they share cassette homology. The imprint could be converted into a DSB during standard methods of DNA purification (43,44) and visualized by autoradiography. To determine the efficiency of mating-type switching, a standard iodine staining assay was carried out. Individual colonies were replicated onto EMM supplemented with the appropriate amino acids and then were grown for 3 days at 22°C before being exposed to iodine vapors.

RESULTS

*Sp*Pol4 a unique X-family DNA polymerase in *S.pombe*

The *S.pombe* ORF SPAC2F7.06c coding for a putative 736 amino acids DNA polymerase from the X family was overproduced in fission yeast wild-type cells and purified to near homogeneity as described in Materials and Methods. The protein, expressed as a fusion protein containing a GST-tag at its N-terminus, was purified by gluthathione–Sepharose affinity and phosphocellulose chromatography. After the purification steps a unique polypeptide was observed in the final fraction, identified by Coomassie blue staining after SDS–PAGE analysis, migrating at the expected position for GST-tagged SPAC2F7.06c (~84 kDa) (data not shown). The purified fraction was assayed for DNA polymerase activity on an activated DNA. As expected, the purified fraction was able to catalyze dNTP incorporation in the presence of either

Mg^{2+} (2×10^{-5} pmol/min ng) or Mn^{2+} (9×10^{-6} pmol/min ng) as activating divalent metal ions. As a control of specificity, we carried out a parallel purification of a catalytically inactive mutant (see Materials and Methods). In this case, no DNA polymerization activity was detectable in the final fraction (data not shown). Therefore, SPAC2F7.06c codifies for a DNA polymerase that we refer to as *Sp*Pol4.

*Sp*Pol4 is a distributive polymerase that lacks 3′→ 5′ exonuclease activity

Processivity is a common feature of DNA polymerases involved in extensive DNA synthesis (i.e. replicative polymerases), and relies on a tight DNA binding and an 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. Distributive polymerization is a common feature of all DNA polymerases from the X family (16,25,35,36,45,46). We assessed *Sp*Pol4 processivity on a DNA template/primer substrate by analyzing the chain length distribution at several enzyme/DNA substrate ratios. As shown in Supplementary Figure 1, the length of the elongated primer decreased with the enzyme/DNA substrate ratio in agreement with a fully distributive polymerization pattern. This distributive behavior of *Sp*Pol4 is also maintained using Mn^{2+} as metal activator (data not shown). Therefore, we conclude that *Sp*Pol4 is a distributive polymerase suited for short-stretch DNA synthesis.

Another distinctive feature of replicative DNA polymerases is its proofreading 3′→5′ exonuclease activity. Three conserved amino acid motifs, named Exo I, Exo II and Exo III, are responsible for the 3′→5′ exonuclease active site of all proofreading DNA polymerases (47). However, these motifs are absent in the DNA polymerases from the X family including *Sp*Pol4, suggesting that, as other PolX enzymes, *Sp*Pol4 has no proofreading activity. We tested this prediction using either a single-stranded oligonucleotide or a template/primer as substrates for 3′→5′ exonucleolysis. Purified *Sp*Pol4 failed to display any nucleolytic activity on both substrates after 15 min at 30°C (data not shown). This result demonstrates that *Sp*Pol4 does not possess 3′→5′ proofreading activity.

*Sp*Pol4 prefers small gaps with a 5′-phosphate group

To further characterize the DNA polymerization activity present in the purified *Sp*Pol4 fraction, we tested different *in vitro* assay conditions using defined templated-DNA molecules in the presence of Mg^{2+} as a cofactor. The purified protein was able to catalyze dNTP incorporation very efficiently either in a template/primer (data not shown) or in the 1 nt gapped substrates in a dNTP dosage-dependent manner (Figure 1A and B). However, a significant increase (10-fold as an average) in the polymerization capacity was observed when a phosphate group was present at the 5′-side of the gap compared with the same gapped DNA molecule having a hydroxyl group at the 5′ end of the gap (Figure 1B). Therefore, the DNA substrate preference of *Sp*Pol4, small gaps with a 5′-phosphate group, is compatible with a role in DNA repair.

In Polβ and Polλ a 5′-phosphate-dependent increase in processivity is structurally and functionally related to the presence of the N-terminal 8 kDa domain (5,7,9,48). Since *Sp*Pol4 also contains an N-terminal 8 kDa domain, and is stimulated by the presence of a 5′-phosphate group in the 1 nt gapped substrate, we tested if this stimulation was primarily due to differences in the DNA-binding capacity, a step preceding dNTP binding

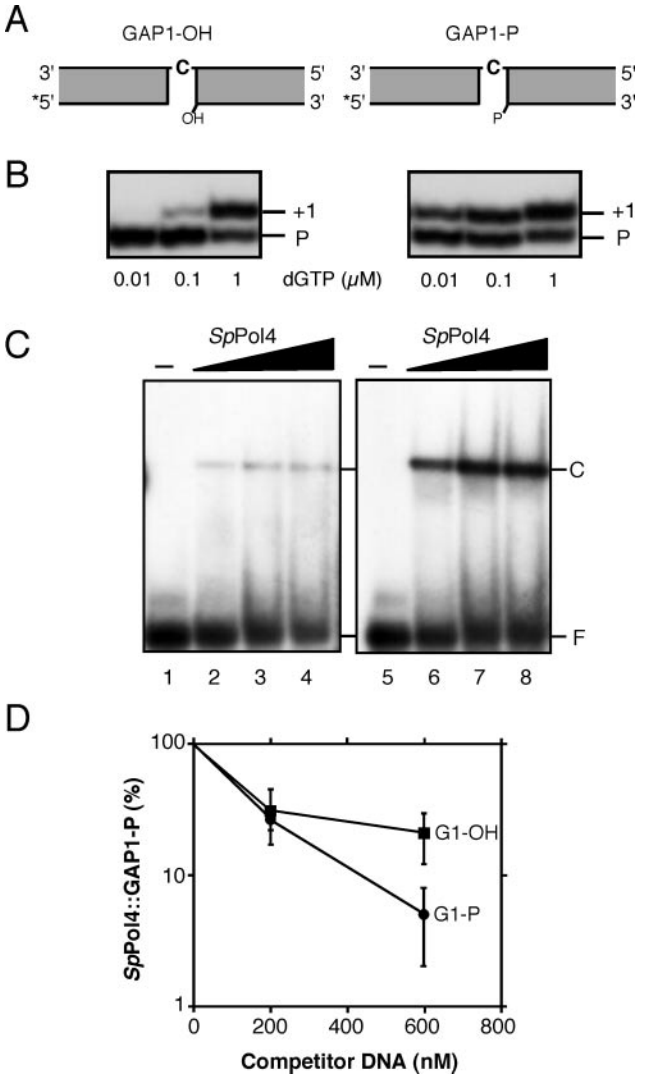

Figure 1. Gap-filling synthesis and substrate preferences of *Sp*Pol4. (**A**) Scheme of the two types of DNA molecules used: 1 nt gap (GAP1-OH) and 1 nt gap with a 5′-phosphate (GAP1-P). Labeled primers (asterisk) and the 5′ end hydroxyl (OH) or phosphate (P) groups are indicated. The oligonucleotides used were T13(C)/SP1C/DG1. (**B**) Gap-filling DNA synthesis of *Sp*Pol4 on the 1 nt gapped structures described above. Reactions were carried out as described in Materials and Methods using 125 nM *Sp*Pol4 and the indicated concentrations of dGTP. Primer extension was analyzed by 8 M urea–PAGE and autoradiography. Mobility of the unextended primer (P) and the 1 nt (+1) extended primers are indicated at the autoradiograph. (**C**) DNA-binding capacity of *Sp*Pol4 to the 1 nt gapped molecules. EMSA was performed as described in Materials and Methods using none (lanes 1 and 5), 250 nM (lanes 2 and 6), 600 nM (lanes 3 and 7) and 1.2 μM (lanes 4 and 8) *Sp*Pol4. Formation of *Sp*Pol4:DNA complexes was resolved by native gel electrophoresis on a 4% polyacrylamide gel (80:1; monomer/bis). Mobility of the free DNA (F) and the *Sp*Pol4/DNA complexes (C) are indicated at the autoradiograph. (**D**) Competition analysis of *Sp*Pol4 bound to the GAP1-P. EMSA was performed as described in Materials and Methods, but using the indicated amounts of either 1 nt gapped 5′-phosphate (G1-P) or 5′-hydroxyl (G1-OH) as unlabeled competitor DNA. The plotted values represent the percentage of labeled GAP1-P that remains bound to *Sp*Pol4 after competition, and are the mean of four independent experiments.

and catalysis. The formation of stable *Sp*Pol4/DNA complexes, assessed by EMSA, required a lower enzyme concentration when the 1 nt gapped DNA had a 5′-phosphate group (Figure 1C). Even more, the affinity of *Sp*Pol4 for the 5′-phosphate group is so strong that when the primer strand is removed, *Sp*Pol4 still binds this molecule almost with the same efficiency (data not shown).

To further analyze the stabilizing effect of the 5′-phosphate on DNA binding by *Sp*Pol4, competition analysis were carried out as indicated in Materials and Methods. As expected, the amount of *Sp*Pol4::GAP1-P (labeled) complexes formed in a previous step progressively decreased when increasing amounts of unlabeled competitor DNA (either having a 5′-phosphate or not) were added, being greater the competition with unlabeled GAP1-P (Figure 1D). However, even at 150-fold molar excess of the 1 nt gapped 5′-phosphate competitor, the amount of *Sp*Pol4::GAP1-P labeled complexes was reduced only by 5% in comparison with that in the absence of the competitor (Figure 1D). Taken together, these results clearly demonstrate that *Sp*Pol4 binds stably and preferentially to a 5′-phosphate containing DNA gap.

*Sp*Pol4 is a template-instructed polymerase with preference for purines

We evaluated the ability of *Sp*Pol4 to discriminate among the four dNTPs in order to catalyze template-directed DNA synthesis. We used a set of 1 nt gapped template-primer substrates with each of the four (X = A, C, G or T) bases as template and having a 5′-phosphate flanking the gap (Figure 2A). For each substrate, the four dNTPs, one complementary to the template and the other three non-complementary, were assayed individually at different concentrations. As shown in Figure 2B, on the four 1 nt gapped substrates, *Sp*Pol4 preferentially incorporated the nucleotide complementary to the first template base, even when non-complementary nucleotides were provided at a 100-fold higher concentration. Therefore, these results suggest that *Sp*Pol4 is template-instructed, i.e. it performs DNA synthesis following the Watson–Crick base pairing rules. Interestingly, quantification of the efficiency of incorporation of each complementary dNTP demonstrated a strong imbalance in correct dNTP incorporation with preference for purines: dG>>dA>dT>dC (Figure 2C).

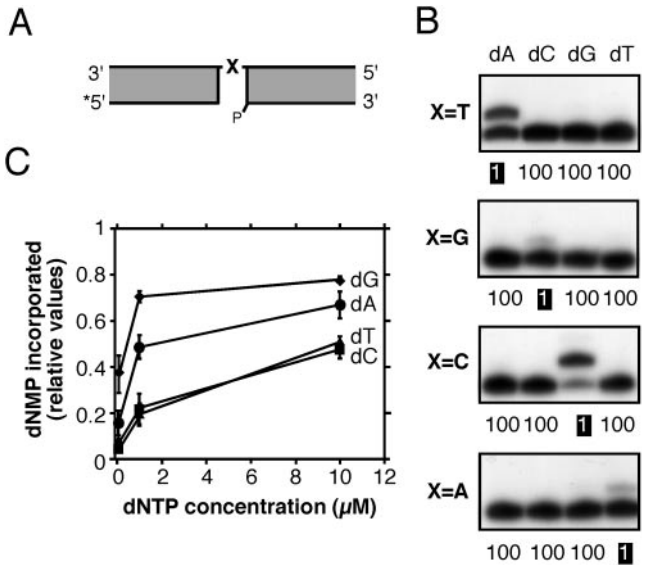

Figure 2. *Sp*Pol4 preferentially incorporates complementary nucleotides. (**A**) Scheme representing the set of 1 nt gapped substrates with a 5′ phosphate group (P) used in this assay, only differing in the templating base (X). The primer strand was 5′ end labeled (asterisk). The oligonucleotides used to obtain these substrates were T32 (A, C, G or T)/P15/DG16. (**B**) Single nucleotide gap-filling assays using any of the four 1 nt gapped DNA substrates, and the four dNTPs (dA, dC, dG and dT) individually provided. Reactions were carried out as described in Materials and Methods using 125 nM *Sp*Pol4. Extension of the labeled primer strand in the presence of either the correct (1 μM) or the incorrect (100 μM) dNTP was analyzed by 8 M urea–20% PAGE and autoradiography. (**C**) Quantification of the complementary dNMP incorporation for the four 1 nt gapped molecules at different dNTP concentrations. The values plotted represent the ratio between the amounts of extended versus total primers, and are the mean of four independent experiments.

Template dislocation and primer realignment capacities of *Sp*Pol4

Human Polμ behaves as an error-prone DNA polymerase, since it is able to induce/accept dislocations of the template strand (35), which is likely crucial for its NHEJ function. To examine whether *Sp*Pol4 is similarly error-prone, we analyzed DNA synthesis in some template sequence contexts that are appropriate for evaluating: (i) slippage-mediated dislocation (Figure 3A); (ii) dNTP selection-mediated dislocation (Figure 3B); and (iii) primer realignment versus direct mismatch extension (Figure 3C). Each dNTP was provided individually to identify the opted mechanism for each DNA polymerase. In the substrate with the dA-track repeat, normal DNA synthesis would lead to dT incorporation, whereas slippage of the primer terminus (dT) to the next template base (dA) would result in dG incorporation and a −1 frameshift DNA synthesis (see schematic representation in Figure 3A). Unlike Polμ, which clearly preferred to insert dG by a slippage-mediated dislocation mechanism, *Sp*Pol4 and Polλ predominantly incorporated dT (Figure 3A). However, both polymerases also incorporated dG and therefore, they can misalign the template-primer to some extent. As shown in Figure 3B, changing the third dA of the track for a dG reduces the possibility of template slippage; therefore, DNA synthesis is more restricted to the canonical incorporation of dC, like *Sp*Pol4 and Polλ do. Besides this normal DNA incorporation event, only Polμ was also able to insert the complementary base (dG) to the position +2 in the template (dC). As reported earlier for Polμ, the template dislocation requirement would be stabilized by the incoming dNTP (49).

To examine the mismatch extension capacity of *Sp*Pol4, we performed a primer extension assay starting from a dA:dC base pair mismatch (see schematic representation in Figure 3C). Because *Sp*Pol4 and other PolX enzymes cannot remove mismatched nucleotides at the primer 3′ end, only two outcomes are possible: (i) direct mismatch extension inserting dC; and (ii) primer terminus realignment inserting dG. As can be seen in Figure 3C, Polλ uses both alternatives almost equally well. In agreement with its extreme error-proneness, Polμ is able to extend the mismatch with any of the four dNTPs. Interestingly, *Sp*Pol4 has a more restricted behavior, as it is only able to insert dG, indicating a significant primer realignment capacity that enables this enzyme for a role in NHEJ.

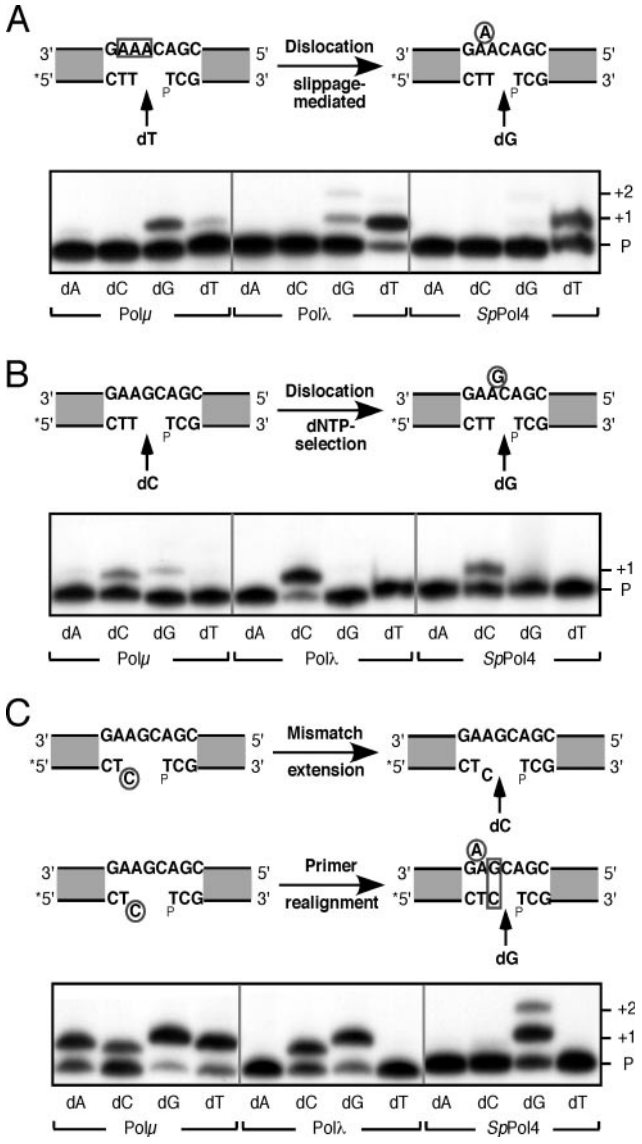

Figure 3. Characterization of *Sp*Pol4 template dislocation and primer realignment capacities at gapped DNA intermediates. Schemes representing template sequence contexts that are appropriate to evaluate slippage-mediated dislocation (**A**), dNTP selection-mediated dislocation (**B**), and primer realignment versus direct mismatch extension (**C**) are shown (see text for details). Labeled primer (asterisk) and 5′ end phosphate group (P) are indicated. Transiently misaligned or mispaired nucleotides are indicated inside a circle, and nucleotide tracks inside a box. A correctly paired primer terminus is indicated inside a box. Polymerization assays were carried out as described in Materials and Methods using 250 nM either *Sp*Pol4, human Polμ or human Polλ, in the presence of each individual dNTP either at 10 μM in all cases (C), or at a different concentration for each DNA polymerase: Polμ (500 nM), Polλ (100 nM) and *Sp*Pol4 (5 μM) (A and B). Primer extension was analyzed by 8 M urea–20% PAGE and autoradiography. Mobility of the unextended primer (P) and the 1 nt (+1) and the 2 nt (+2) extended primers are indicated at the autoradiographs.

*Sp*Pol4 inserts both rNTPs and dNTPs with the same efficiency

Polμ and TdT have the striking ability to incorporate both rNTPs and dNTPs to nucleic acid chains (36,37,45). This unusual capacity mainly relies on a single glycine residue that opens the 'steric gate', which is frequently closed by a conserved aromatic residue present in Polβ, Polλ and in other members of the PolX family of DNA-dependent DNA polymerases (Figure 4A) (36). Since *Sp*Pol4 also has a glycine residue at this position (Gly^{434}), it seemed very likely that *Sp*Pol4 could incorporate rNTPs. By using the same four 1 nt gapped template–primer substrates as in Figure 2B, it was shown that *Sp*Pol4 efficiently incorporates rNTPs on a DNA primer strand (Figure 4B) with almost equal efficiency as dNTPs and displaying the same preference pattern for purines (compare Figures 4B and 2C).

Using a competition assay in which both sugars (ribose and deoxyribose) are simultaneously provided, the sugar selectivity factor of a given DNA polymerase can be calculated (36,45). Since the rNTP and the dNTP have different molecular weights, the +1 extended primers can be easily separated by gel electrophoresis and quantified. The sugar selectivity factor is given as the ratio between the amounts of primer extended with rNTP versus dNTP. Irrespective of the nature of the base, the sugar selectivity factors obtained for *Sp*Pol4 were very similar (0.74–0.91) and proximal to one, indicating a lack of discrimination between rNTPs and dNTPs (Figure 4C). These values are similar to those obtained for TdT (43) and Polμ (36) in untemplated and templated reactions, respectively, being several orders of magnitude smaller than the ones reported for other DNA polymerases (50–52).

*Sp*Pol4 is not required for imprinting at the *mat1 locus*

It is known for many years that mating-type switching in *S.pombe* depends on a strand-specific imprint at the *mat1 locus* (53,54). The imprint was characterized either as an alkali-labile modification or as a nick that could be converted into a DSB during standard methods of DNA purification (43,44). More recently, this imprint has been characterized as an RNase-sensitive modification that consists of one or two RNA residues incorporated into the *mat1 locus* (55). Based on these results, it was tempting to speculate with the possibility that *Sp*Pol4 might be responsible at the incorporation of these one or two RNA residues. *S.pombe* genomic DNA was prepared by a standard yeast extraction protocol (see Materials and Methods), digested with HindIII and analyzed by Southern hybridization using a 1 kb *mat1-P* as a probe. The h^{90} wild-type strain yielded the typical bands of uncleaved (10.4 kb; *mat1*) and cleaved (5.4 kb; *mat1**) products, together with two other bands representing cross-hybridization of the *mat1-P* probe with the *mat2* (6.3 kb) and *mat3* (4.2 kb) *loci* (Supplementary Figure 2A and B, lane 2). As expected, the *smt-0* mutant strain (56), containing a deletion of the *cis*-acting elements SAS1 and SAS2 and thus preserving the integrity of the cleavage site sequence while abolishing mating-type switching, did not produce the *mat1** band (57) (Supplementary Figure 2B, lane 1). However, the amount of cleavage at the *mat1 locus*, represented by the 5.4 kb (*mat1**) band, obtained in the h^{90} *pol4*Δ mutant was the same as the level obtained in the h^{90} wild-type strain (Supplementary Figure 2B, compare lanes 2 and 3). Moreover, direct measurement of the mating-type switching efficiency by the iodine staining assay (see Materials and Methods) was carried out. The starch reaction with iodine vapors stains spore-containing colonies black, whereas slow-switching mutants exhibit streaky iodine staining patterns and colonies unable to switch

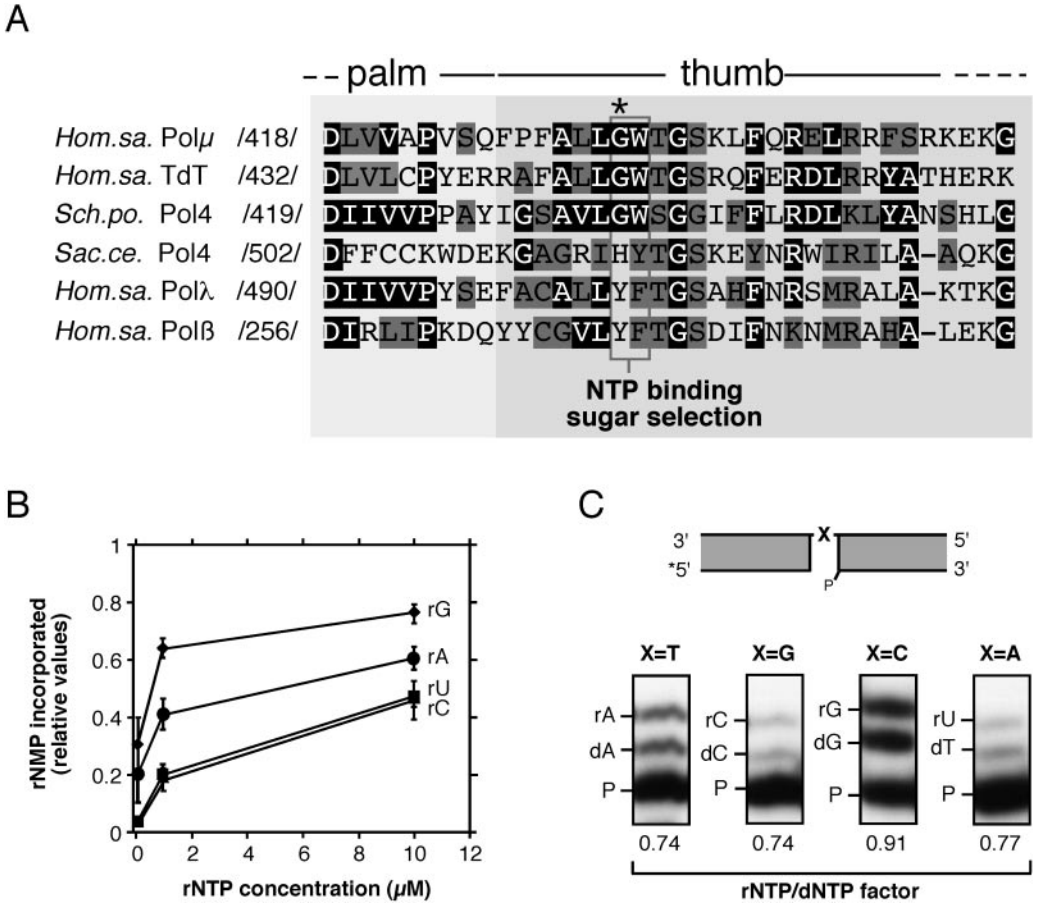

Figure 4. Lack of sugar discrimination by *Sp*Pol4. (**A**) Multiple amino acid alignment of the amino acid region (connecting subdomains palm and thumb) probably involved in sugar discrimination in the Pol X family. Numbers between slashes indicate the amino acid position relative to the N-terminus of each polymerase. Invariant (in white letters over a black background) and conservative substitutions referred to *Sp*Pol4 residues are boxed in dark gray. The two amino acid residues most probably involved in sugar discrimination are inside a box, and the Gly residue critical for rNTP incorporation is indicated with an asterisk. Abbreviations used are: Hom.sa. (*Homo sapiens*), Sch.po. (*S.pombe*), and Sac.ce. (*S.cerevisiae*). (**B**) *Sp*Pol4 inserts rNTPs efficiently. The assay, essentially as described in Figure 2, evaluates complementary rNMP incorporation into the four 1 nt gapped molecules, at different rNTP concentrations. The plotted values represent the ratio between the amount of extended versus total primers, and are the mean of four independent experiments. (**C**) Lack of sugar discrimination by *Sp*Pol4. The four 1 nt gapped DNA substrates described in Figure 2, differing in the templating base (X) (a scheme is shown), were used. In this competition assay, each complementary duo of nucleotides (rNTP + dNTP) was simultaneously provided at 100 nM. Reactions were carried out as described in Materials and Methods using 125 nM *Sp*Pol4. Mobility of the primer (P) and the rNTP (rN) or dNTP (dN) extended primers was analyzed by 8 M urea–20% PAGE and autoradiograph. The sugar selectivity factor is given as the ratio between the amount of rNTP- versus dNTP-extended primers.

the mating type appear yellowish. As expected, iodine vapors stained the *smt-0* mutant colonies yellowish, whereas the h^{90} wild-type and the *pol4*Δ colonies were stained black (data not shown). Therefore, the imprint at *mat1* remains unaffected in the absence of *Sp*Pol4 and if there were one or two RNA residues in the DNA, the incorporation would be *Sp*Pol4-independent.

*Sp*Pol4 has no TdT activity

Polμ, as TdT, displays an intrinsic deoxynucleotidyltransferase activity, which is stronger in the presence of Mn^{2+} as cofactor (33). This enzymatic activity requires a region of the palm subdomain called loop1, which is absent in *Sp*Pol4, Polβ and Polλ (Supplementary Figure 3A). It has been demonstrated that the deletion of this loop abolishes the TdT-like activity of human Polμ (29) (R. Juárez, J. F. Ruiz, S. A. Nick McElhinny, D. A. Ramsden and L. Blanco, manuscript submitted). TdT activity can only be unambiguously determined by using single-stranded homopolymeric DNA as primer and any of the three dNTPs not included in the primer sequence. Thus, using a ^{32}P-labeled 15mer dT oligonucleotide, in the presence of either Mg^{2+} or Mn^{2+}, *Sp*Pol4 displayed no TdT activity (Supplementary Figure 3B).

*Sp*Pol4 has an intrinsic dRP lyase activity most probably involved in BER

The amino acid residues that are critical for dRPase activity are conserved in the 8 kDa domain of Polβ and Polλ (18,24) and are indicated with dots in Figure 5A. Among them, a specific lysine (Lys^{72} in Polβ and Lys^{312} in Polλ) is the catalytic residue acting as a Schiff-base during β-elimination of the dRP moiety. As shown in Figure 5A, Polμ and *Sp*Pol4 lack the catalytic lysine residue, and as it has been demonstrated for Polμ (24), it was probable that *Sp*Pol4 was devoid of dRP lyase activity. However, by using standard BER assays (18,24), here we show that *Sp*Pol4 is able to remove a dRP group and

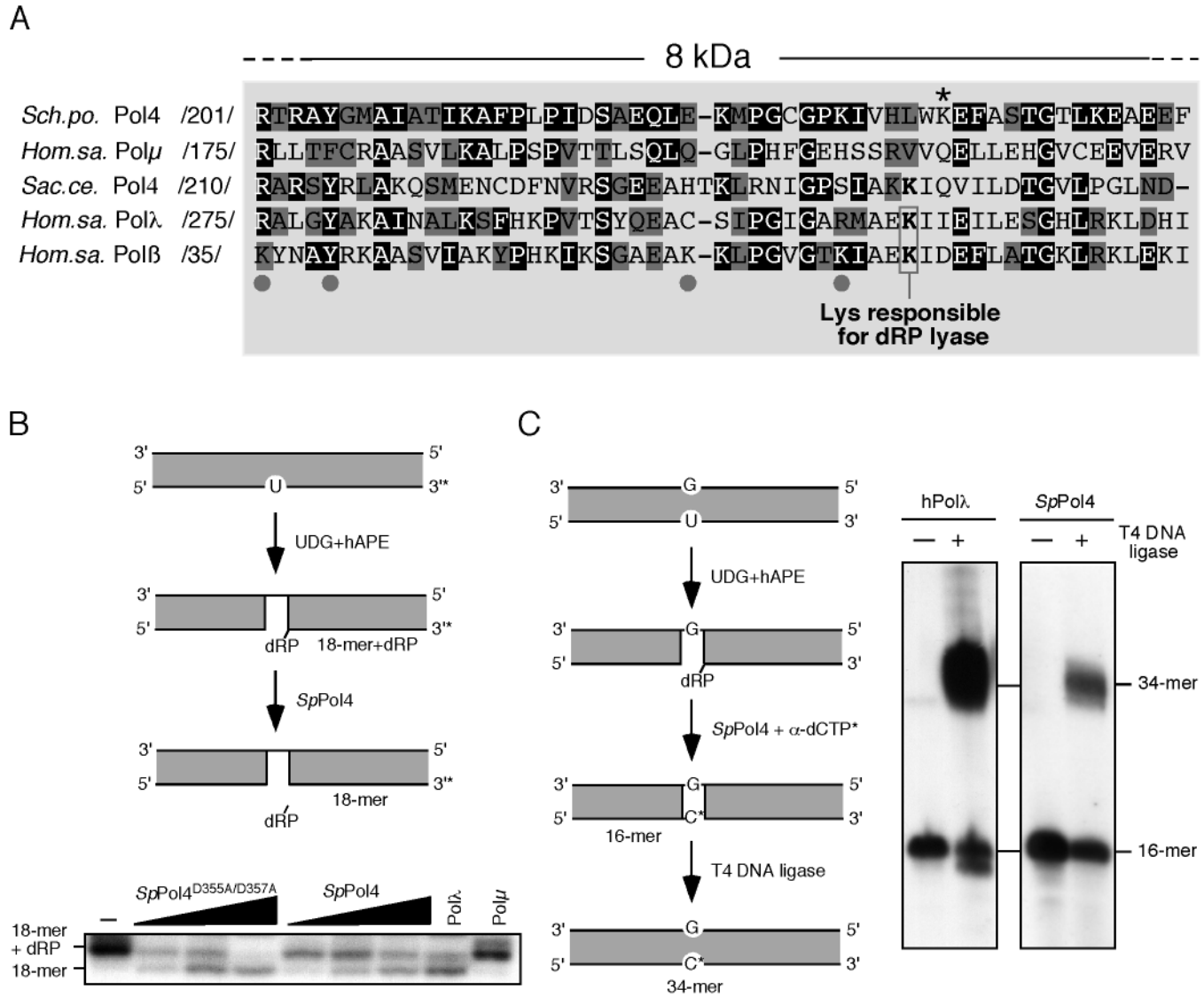

Figure 5. Characterization of *Sp*Pol4 dRP lyase activity and reconstitution of BER *in vitro*. (**A**) Multiple amino acid alignment of the 8 kDa domain of *Sp*Pol4 with other family X DNA polymerases members. Numbers between slashes indicate the amino acid position relative to the N-terminus of each polymerase. Residues described to be relevant to the dRP lyase function of *Hs*Polβ (Lys^{35}, Tyr^{39}, Lys^{60}, Lys^{68}) are indicated with dots. Residues that are essential for (*Hs*Polβ Lys^{72}; *Hs*Polλ Lys^{312}) or may be involved in (*Sc*Pol4 Lys^{248}) dRP lyase activity are in bold type. The position of *Sp*Pol4 Lys^{240} that might substitute for *Hs*Polβ Lys^{72} is indicated with an asterisk. Invariant (in white letters over a black background) and conservative substitutions referred to *Sp*Pol4 residues are boxed in dark gray. Abbreviations used are: Hom.sa. (*H.sapiens*), Sch.po. (*S.pombe*), and Sac.ce. (*S.cerevisiae*). (**B**) *In vitro* analysis of the dRP lyase reaction. The scheme shows a 34mer double-stranded oligonucleotide containing an uracil residue (at position 16) in the strand which is 3′ end labeled (asterisk). After treatment with UDG and hAPE, a dRP-containing nicked substrate (18mer+dRP) is obtained. As shown in the autoradiogram, the dRP moiety can be cleaved by incubation with either human Polλ (60 nM), wild-type *Sp*Pol4 or *Sp*Pol4$^{D355A/D357A}$ (20, 60 and 120 nM), as the labeled strand is detected as an 18mer product after denaturing electrophoresis. Human Polμ (70 nM) was included as a negative control, lacking dRP lyase activity. (**C**) *In vitro* reconstitution of a BER reaction with *Sp*Pol4. A 34mer double-stranded oligonucleotide containing an uracil residue at position 16 in one strand is treated with UDG (100 nM) and hAPE (40 nM) to release a dRP-containing nicked substrate. By adding a labeled dNTP (α-dCTP) and either purified human Polλ (245 nM) or *Sp*Pol4 (125 nM), two labeled products can be observed after denaturing electrophoresis and autoradiography: (i) a 16mer product generated by a single nucleotide insertion at the 3′-hydroxyl end of the 5′-incised AP site; (ii) a 34mer product that corresponds to the complete repair of the DNA strand upon T4 DNA ligase action.

promote single-patch BER *in vitro*. By adding UDG and hAPE to a uracil-containing substrate, a nicked strand with a 3-hydroxyl and a 5′-dRP is produced. The strand containing the 5′-dRP moiety, which is 3′ end labeled, migrates at the expected position of an 18mer + dRP (Figure 5B). By using increasing amounts of *Sp*Pol4, this product was converted to a shorter product (18mer), indicating that *Sp*Pol4 as Polλ, and unlike Polμ, has an intrinsic dRP lyase activity (Figure 5B). Similarly, the *Sp*Pol4$^{D355A/D357A}$ polymerization-deficient mutant was proficient in dRP lyase activity similar to the wild-type *Sp*Pol4 (Figure 5B). Unexpectedly from the alignment shown in Figure 5A, *Sp*Pol4 has dRP lyase activity though it lacks the lysine residue conserved in Polβ (Lys^{72}) and Polλ (Lys^{312}) responsible for their dRP lyase activities (24,58).

Removal of a dRP residue is an essential step for the completion of single nucleotide BER. Polβ and Polλ are able to efficiently promote *in vitro* BER of a uracil-containing duplex

DNA in the presence of hUDG, hAPE and DNA ligase I (24,59,60). As shown in Figure 5C, two main products were observed in a human Polλ-based reconstituted BER reaction: a 16mer product generated by a single nucleotide insertion (dCTP labeled) at the 3′-hydroxyl end of the 5′-incised AP site, and a 34mer product that corresponds to the complete repair of the DNA strand upon DNA ligase action. As shown in Figure 5C, both *Sp*Pol4 and hPolλ are able to produce the same 16 and 34mer labeled products. Thus, *Sp*Pol4 is able to coordinate both the gap-filling and dRP excision steps of repair preceding DNA ligase action. These data are consistent with a role for *Sp*Pol4 in BER and predict that the dRP lyase-containing enzyme *Sp*Pol4 could participate in BER *in vivo*.

DISCUSSION

In mammals, there are five members belonging to the X family of DNA polymerases: Polβ, Polλ, Polμ, Polσ and TdT. On the contrary, yeasts, plants, and some bacteria and viruses have only one PolX enzyme (3). In *S.pombe*, the entry SPAC2F7.06c (GeneDB http://www.genedb.org/) predicted a putative DNA PolX as inferred by sequence comparison analysis. Based on the results presented here, it can be concluded that SPAC2F7.06c does codify for a novel DNA polymerase belonging to the PolX family that would be adequately designated as *Sp*Pol4.

The structural organization of *Sp*Pol4 as an N-terminal BRCT domain followed by a C-terminal 39 kDa Polβ-like core domain resembles members of the family X DNA polymerases, such as Polμ, TdT and Polλ. Excluding the more variable N-terminal BRCT domain (61), *Sp*Pol4 is more closely related to Polμ (27% identical core residues), followed by Polλ (24% identity) and Polβ (20% identity). It is worth noting that budding yeast has also one DNA PolX (*Sc*Pol4) that, unlike *Sp*Pol4, resembles Polλ in its core domain (25% identity) and in its structural organization (62). Therefore, based only on their coding sequences, it was speculated that *Sp*Pol4 is a yeast orthologue of Polμ, whereas *Sc*Pol4 would be an orthologue of Polλ (3).

As summarized in Table 1, our biochemical analysis demonstrated that *Sp*Pol4 is capable of carrying out DNA synthesis in a template-dependent manner and exhibits low processivity during primer extension. Such properties are shared by all members of the eukaryotic X family, except TdT [reviewed in (63)]. EMSAs showed that *Sp*Pol4 binds to 5′-phosphate gapped substrates better than to those with a 5′-hydroxyl, and this should imply an improvement in polymerization on the former substrates. As indicated in Table 1, the improved polymerization activity dependent on a 5′-phosphate group described here for *Sp*Pol4 is also an attribute of Polβ and Polλ (23), and Polμ (R. Juárez, P. Andrade and L. Blanco, unpublished data), but not of *Sc*PolIV (64).

Most residues involved in dRP lyase activity are conserved between Polβ and Polλ (24). Among them, the nucleophile residue at position Lys^{72} (Polβ) or Lys^{312} (Polλ), responsible for 90% of the activity (18,24), is conserved in *Sc*Pol4 (Lys^{248}), but not in Polμ and TdT (which lack dRP lyase activity), and is also absent in *Sp*Pol4. Unexpectedly, *Sp*Pol4 was shown to have dRP lyase activity although it lacks this conserved residue. Nonetheless, other residues proposed in Polβ to facilitate removal of the dRP group are indeed present in *Sp*Pol4, and an alternative lysine (Lys^{240}) could be acting as the attacking nucleophile (for details see Figure 5). In any case, and based on our *in vitro* assays, we propose that *Sp*Pol4 could play a role in BER, as Polβ and Polλ. Based on the demonstration of an intrinsic dRP lyase activity, a similar role for *Sc*Pol4 has been proposed recently (64).

As shown in this paper, the relative nucleotide usage of *Sp*Pol4 is different from that observed for other DNA-dependent DNA polymerases of the X family (Polβ, Polλ and Polμ). In particular, *Sp*Pol4 preferentially inserts purine nucleotides in the following order: dG>>dA>dT>dC. Hydrolysis, alkylation, oxidation and deamination are the major forms of DNA damage in all living cells, which are mainly repaired by BER. It is worth noting that, at least in mammalian cells, purines are lost 20-fold more frequently than pyrimidines (∼10 000/cell/day versus ∼500/cell/day, respectively). Additionally, purines are the most frequently alkylated bases and guanine is the base more prone to oxidation, resulting in 8-oxoG (100–1000/cell/day) and along with adenine in a ring-opened form called formamidopyrimidine (FaPyG and FaPyA). Only deamination, another prevalent form of DNA damage, occurs predominately at cytosine, turning it into uracil (100–500/cell/hour) (1,21). Therefore, it is tempting to speculate that the preference of *Sp*Pol4 for purine nucleotides has been adapted to cope with a more intensive role of repairing purine bases.

In addition to its preference for small gaps, the unusual capacity of *Sp*Pol4 to accept misaligned template–primer molecules as a substrate and to realign 3′-terminal mismatches would be very convenient for microhomology-mediated NHEJ. Moreover, some BER intermediates, as staggered nicks made by an AP endonuclease in opposite strands, originate DSBs that would trigger the NHEJ pathway. Under these circumstances, a DNA repair polymerase endowed with dRP lyase activity would be very convenient to process the damaged DNA ends and eliminate the dRP residues. Physical and functional interactions with factors of NHEJ have been reported for Polμ, Polλ and *Sc*Pol4 (27,38,39,41), occurring through the BRCT domain of these proteins. The presence of a BRCT domain at the N-terminus of *Sp*Pol4 would support similar interactions with NHEJ factors operating in *S.pombe*.

Most DNA polymerases have an exquisite sugar selectivity and prefer to incorporate dNTPs over rNTPs by a factor of 10^4- to 10^6-fold (50). Sugar discrimination has been shown to

Table 1. Comparison of *Sp*Pol4 properties to other template-dependent members of the DNA PolX family

DNA PolX	Stimulation by 5′-P	Dislocation by slippage	Dislocation by dNTP selection	rNTP usage	Terminal transferase	dRP lyase
*Sp*Pol4	Yes	Yes	weak	Yes	No	Yes
*Sc*Pol4[a]	No	Strong	weak	Yes	?	Yes
Polλ	Yes	Strong	weak	No	weak	Yes
Polμ	Yes	Very strong	Strong	Yes	Yes	No
Polβ	Yes	Yes	weak	No	No	Yes

[a]Taken from (64).

depend on a steric barrier for the 2′-hydroxyl of an incoming rNTP (4,50,65). Accordingly, Polβ and Polλ are unable to incorporate rNTP since they have bulky residues close to the 2′ position of the ribose of the incoming nucleotide (Tyr^{271}-Phe^{272} and Tyr^{505}-Phe^{506}, respectively). However, Polμ and TdT, which efficiently insert rNTPs (36,37,45), have a small residue in the pair (Gly^{433}-Trp^{434} and Gly^{448}-Trp^{449}) that was shown to be responsible for rNTP insertion (34). As shown here, *Sp*Pol4 resembles Polμ and TdT, as it also incorporates rNTP very efficiently. This property was expected because the two residues equivalent to Polμ (Gly^{433}-Trp^{434}) are strictly conserved in *Sp*Pol4 (Gly^{434}-Trp^{435}). Strikingly, it has been recently reported that *Sc*Pol4, although having two aromatic residue at these positions (His^{517}-Tyr^{518}) also incorporates rNTPs with a high efficiency (64).

It has been demonstrated that NHEJ is a predominant repair pathway in G_1 phase and probably in non-cycling cells (66–68). In contrast to dNTPs, abundant during S phase, rNTPs are available at high levels in all phases of the cell cycle (69,70). Therefore, as suggested for human Polμ (38) and ScPol4 (64), the extraordinary ability of *Sp*Pol4 to incorporate rNTPs would be very convenient for a role in NHEJ. Moreover, insertion of rNTPs might also be useful in BER to repair modified or damaged bases into DNA throughout the cell cycle that could be removed by the sequential action of RNaseH35/RNaseH type II and Rad27/FEN-1 (71). Further work should be carried out to ascertain this specific pathway in *S.pombe*.

It has been reported that the imprinting step during mating-type switching in *S.pombe* is an RNase-sensitive modification that consists of one or two RNA residues incorporated into the *mat1 locus* (55), which becomes a fragile chromosome site. Taking into account the capacity of *Sp*Pol4 to incorporate a few rNTPs in the DNA, it was tempting to speculate with a probable involvement of *Sp*Pol4 in mating-type switching in *S.pombe*. As shown here, the lack of *Sp*Pol4 (h^{90} *pol4*Δ strain) did not affect either the level of DSBs in the *mat1 locus* or the mating-type efficiency with respect to the h^{90} wild-type strain; therefore, we conclude that the imprint should remain unaffected. Thus, if there were some RNA residues in the *mat1 locus*, the incorporation would be *Sp*Pol4-independent. Alternatively, the imprint could imply a strand-specific nick with no flanking RNA residues (72).

In conclusion, the results presented here demonstrate that *Sp*Pol4 shares biochemical properties with different members of the PolX super-family; thus, it must be considered to be a unique enzyme (see Table 1 for a comparison). Mammalian PolXs became specialized to play a role in BER (Polβ), in NHEJ coupled with BER (Polλ), microhomology-mediated NHEJ (Polμ) or V(D)J recombination (TdT). However, the fact that both fission and budding yeasts had only one DNA Pol X suggests that they are evolutionarily closer to the stem ancestor of the family, which is also consistent with a less specialized and multipotential role in different forms of DNA repair, enabled by a combination of the biochemical properties of their mammalian homologues.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

We thank Juan Jiménez for *S.pombe* strains and plasmids and Aurelia Lahoz for her inestimable advice and help with *S.pombe* handling and techniques. This work was supported by Ministerio de Ciencia y Tecnología Grant BMC 2003-00186, and by an institutional grant to Centro de Biología Molecular 'Severo Ochoa' from Fundación Ramón Areces. A.S., R.J., A.J.P. and G.T. were recipients of a fellowship from the Ministerio de Educación y Ciencia. Funding to pay the Open Access publication charges for this article was provided by the Spanish Ministry of Science and Technology.

Conflict of interest statement. None declared.

REFERENCES

1. Lindahl,T. and Wood,R.D. (1999) Quality control by DNA repair. *Science*, **286**, 1897–1905.
2. Boiteux,S. and Guillet,M. (2004) Abasic sites in DNA: repair and biological consequences in *Saccharomyces cerevisiae*. *DNA Repair (Amst.)*, **3**, 1–12.
3. Burgers,P.M., Koonin,E.V., Bruford,E., Blanco,L., Burtis,K.C., Christman,M.F., Copeland,W.C., Friedberg,E.C., Hanaoka,F., Hinkle,D.C. *et al*. (2001) Eukaryotic DNA polymerases: proposal for a revised nomenclature. *J. Biol. Chem*., **276**, 43487–43490.
4. Aravind,L. and Koonin,E.V. (1999) DNA polymerase beta-like nucleotidyltransferase superfamily: identification of three new families, classification and evolutionary history. *Nucleic Acids Res*., **27**, 1609–1618.
5. Hübscher,U., Maga,G. and Spadari,S. (2002) Eukaryotic DNA polymerases. *Annu. Rev. Biochem*., **71**, 133–163.
6. Pelletier,H., Sawaya,M.R., Kumar,A., Wilson,S.H. and Kraut,J. (1994) Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP. *Science*, **264**, 1891–1903.
7. Sawaya,M.R., Pelletier,H., Kumar,A., Wilson,S.H. and Kraut,J. (1994) Crystal structure of rat DNA polymerase beta: evidence for a common polymerase mechanism. *Science*, **264**, 1930–1935.
8. Delarue,M., Boule,J.B., Lescar,J., Expert-Bezancon,N., Jourdan,N., Sukumar,N., Rougeon,F. and Papanicolaou,C. (2002) Crystal structures of a template-independent DNA polymerase: murine terminal deoxynucleotidyltransferase. *EMBO J*., **21**, 427–439.
9. Garcia-Diaz,M., Bebenek,K., Krahn,J.M., Blanco,L., Kunkel,T.A. and Pedersen,L.C. (2004) A structural solution for the DNA polymerase lambda-dependent repair of DNA gaps with minimal homology. *Mol. Cell*, **13**, 561–572.
10. Garcia-Diaz,M., Bebenek,K., Krahn,J.M., Kunkel,T.A. and Pedersen,L.C. (2005) A closed conformation for the Pol lambda catalytic cycle. *Nature Struct. Mol. Biol*., **12**, 97–98.
11. Maciejewski,M.W., Shin,R., Pan,B., Marintchev,A., Denninger,A., Mullen,M.A., Chen,K., Gryk,M.R. and Mullen,G.P. (2001) Solution structure of a viral DNA repair polymerase. *Nature Struct. Biol*., **8**, 936–941.
12. Showalter,A.K., Byeon,I.J., Su,M.I. and Tsai,M.D. (2001) Solution structure of a viral DNA polymerase X and evidence for a mutagenic function. *Nature Struct. Biol*., **8**, 942–946.
13. Bork,P., Hofmann,K., Bucher,P., Neuwald,A.F., Altschul,S.F. and Koonin,E.V. (1997) A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. *FASEB J*., **11**, 68–76.
14. Bebenek,K. and Kunkel,T.A. (2004) Functions of DNA polymerases. *Adv. Protein Chem*., **69**, 137–165.
15. Kunkel,T.A. and Loeb,L.A. (1981) Fidelity of mammalian DNA polymerases. *Science*, **213**, 765–767.
16. Kunkel,T.A. (1985) The mutational specificity of DNA polymerase-beta during *in vitro* DNA synthesis. Production of frameshift, base substitution, and deletion mutations. *J. Biol. Chem*., **260**, 5787–5796.
17. Singhal,R.K. and Wilson,S.H. (1993) Short gap-filling synthesis by DNA polymerase beta is processive. *J. Biol. Chem*., **268**, 15906–15911.
18. Prasad,R., Beard,W.A., Chyan,J.Y., Maciejewski,M.W., Mullen,G.P. and Wilson,S.H. (1998) Functional analysis of the amino-terminal 8-kDa domain of DNA polymerase beta as revealed by site-directed

mutagenesis. DNA binding and 5′-deoxyribose phosphate lyase activities. *J. Biol. Chem.*, **273**, 11121–11126.
19. Singhal,R.K., Prasad,R. and Wilson,S.H. (1995) DNA polymerase beta conducts the gap-filling step in uracil-initiated base excision repair in a bovine testis nuclear extract. *J. Biol. Chem.*, **270**, 949–957.
20. Dianov,G., Price,A. and Lindahl,T. (1992) Generation of single-nucleotide repair patches following excision of uracil residues from DNA. *Mol. Cell. Biol.*, **12**, 1605–1612.
21. Nilsen,H. and Krokan,H.E. (2001) Base excision repair in a network of defence and tolerance. *Carcinogenesis*, **22**, 987–998.
22. Dianov,G.L., Sleeth,K.M., Dianova,I.I. and Allinson,S.L. (2003) Repair of abasic sites in DNA. *Mutat. Res.*, **531**, 157–163.
23. Braithwaite,E.K., Prasad,R., Shock,D.D., Hou,E.W., Beard,W.A. and Wilson,S.H. (2005) DNA polymerase lambda mediates a back-up base excision repair activity in extracts of mouse embryonic fibroblasts. *J. Biol. Chem.*, **280**, 18469–18475.
24. Garcia-Diaz,M., Bebenek,K., Kunkel,T.A. and Blanco,L. (2001) Identification of an intrinsic 5′-deoxyribose-5-phosphate lyase activity in human DNA polymerase lambda: a possible role in base excision repair. *J. Biol. Chem.*, **276**, 34659–34663.
25. Garcia-Diaz,M., Bebenek,K., Sabariegos,R., Dominguez,O., Rodriguez,J., Kirchhoff,T., Garcia-Palomero,E., Picher,A.J., Juarez,R., Ruiz,J.F. *et al.* (2002) DNA polymerase lambda, a novel DNA repair enzyme in human cells. *J. Biol. Chem.*, **277**, 13184–13191.
26. Lee,J.W., Blanco,L., Zhou,T., Garcia-Diaz,M., Bebenek,K., Kunkel,T.A., Wang,Z. and Povirk,L.F. (2004) Implication of DNA polymerase lambda in alignment-based gap filling for nonhomologous DNA end joining in human nuclear extracts. *J. Biol. Chem.*, **279**, 805–811.
27. Ma,Y., Lu,H., Tippin,B., Goodman,M.F., Shimazaki,N., Koiwai,O., Hsieh,C.L., Schwarz,K. and Lieber,M.R. (2004) A biochemically defined system for mammalian nonhomologous DNA end joining. *Mol. Cell*, **16**, 701–713.
28. Fan,W. and Wu,X. (2004) DNA polymerase lambda can elongate on DNA substrates mimicking non-homologous end joining and interact with XRCC4-ligase IV complex. *Biochem. Biophys. Res. Commun.*, **323**, 1328–1333.
29. Nick McElhinny,S.A., Havener,J.M., Garcia-Diaz,M., Juarez,R., Bebenek,K., Kee,B.L., Blanco,L., Kunkel,T.A. and Ramsden,D.A. (2005) A gradient of template dependence defines distinct biological roles for family X polymerases in nonhomologous end joining. *Mol. Cell*, **19**, 1–10.
30. Gilfillan,S., Dierich,A., Lemeur,M., Benoist,C. and Mathis,D. (1993) Mice lacking TdT: mature animals with an immature lymphocyte repertoire. *Science*, **261**, 1175–1178.
31. Komori,T. and Sugiyama,H. (1993) N sequences, P nucleotides and short sequence homologies at junctional sites in VH to VHDJH and VHDJH to JH joining. *Mol. Immunol.*, **30**, 1393–1398.
32. Bertocci,B., De Smet,A., Berek,C., Weill,J.C. and Reynaud,C.A. (2003) Immunoglobulin kappa light chain gene rearrangement is impaired in mice deficient for DNA polymerase mu. *Immunity*, **19**, 203–211.
33. Dominguez,O., Ruiz,J.F., Lain de Lera,T., Garcia-Diaz,M., Gonzalez,M.A., Kirchhoff,T., Martinez,A.C., Bernad,A. and Blanco,L. (2000) DNA polymerase mu (Pol mu), homologous to TdT, could act as a DNA mutator in eukaryotic cells. *EMBO J.*, **19**, 1731–1742.
34. Ruiz,J.F., Dominguez,O., Lain de Lera,T., Garcia-Diaz,M., Bernad,A. and Blanco,L. (2001) DNA polymerase mu, a candidate hypermutase? *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **356**, 99–109.
35. Zhang,Y., Wu,X., Yuan,F., Xie,Z. and Wang,Z. (2001) Highly frequent frameshift DNA synthesis by human DNA polymerase mu. *Mol. Cell. Biol.*, **21**, 7995–8006.
36. Ruiz,J.F., Juarez,R., Garcia-Diaz,M., Terrados,G., Picher,A.J., Gonzalez-Barrera,S., Fernandez de Henestrosa,A.R. and Blanco,L. (2003) Lack of sugar discrimination by human Pol mu requires a single glycine residue. *Nucleic Acids Res.*, **31**, 4441–4449.
37. Nick McElhinny,S.A. and Ramsden,D.A. (2003) Polymerase mu is a DNA-directed DNA/RNA polymerase. *Mol. Cell. Biol.*, **23**, 2309–2315.
38. Mahajan,K.N., Nick McElhinny,S.A., Mitchell,B.S. and Ramsden,D.A. (2002) Association of DNA polymerase mu (pol mu) with Ku and ligase IV: role for pol mu in end-joining double-strand break repair. *Mol. Cell. Biol.*, **22**, 5194–5202.
39. Wilson,T.E. and Lieber,M.R. (1999) Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway. *J. Biol. Chem.*, **274**, 23599–23609.
40. Tseng,H.M. and Tomkinson,A.E. (2002) A physical and functional interaction between yeast Pol4 and Dnl4-Lif1 links DNA synthesis and ligation in nonhomologous end joining. *J. Biol. Chem.*, **277**, 45630–45637.
41. Tseng,H.M. and Tomkinson,A.E. (2004) Processing and joining of DNA ends coordinated by interactions among Dnl4/Lif1, Pol4, and FEN-1. *J. Biol. Chem.*, **279**, 47580–47588.
42. Wach,A., Brachat,A., Pohlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.
43. Dalgaard,J.Z. and Klar,A.J. (1999) Orientation of DNA replication establishes mating-type switching pattern in *S.pombe*. *Nature*, **400**, 181–184.
44. Arcangioli,B. (1998) A site- and strand-specific DNA break confers asymmetric switching potential in fission yeast. *EMBO J.*, **17**, 4503–4510.
45. Boule,J.B., Rougeon,F. and Papanicolaou,C. (2001) Terminal deoxynucleotidyl transferase indiscriminately incorporates ribonucleotides and deoxyribonucleotides. *J. Biol. Chem.*, **276**, 31388–31393.
46. Prasad,R., Widen,S.G., Singhal,R.K., Watkins,J., Prakash,L. and Wilson,S.H. (1993) Yeast open reading frame YCR14C encodes a DNA beta-polymerase-like enzyme. *Nucleic Acids Res.*, **21**, 5301–5307.
47. Bernad,A., Blanco,L., Lazaro,J.M., Martin,G. and Salas,M. (1989) A conserved 3′→5′ exonuclease active site in prokaryotic and eukaryotic DNA polymerases. *Cell*, **59**, 219–228.
48. Prasad,R., Beard,W.A. and Wilson,S.H. (1994) Studies of gapped DNA substrate binding by mammalian DNA polymerase beta. Dependence on 5′-phosphate group. *J. Biol. Chem.*, **269**, 18096–18101.
49. Ruiz,J.F., Lucas,D., Garcia-Palomero,E., Saez,A.I., Gonzalez,M.A., Piris,M.A., Bernad,A. and Blanco,L. (2004) Overexpression of human DNA polymerase mu (Pol mu) in a Burkitt's lymphoma cell line affects the somatic hypermutation rate. *Nucleic Acids Res.*, **32**, 5861–5873.
50. Joyce,C.M. (1997) Choosing the right sugar: how polymerases select a nucleotide substrate. *Proc. Natl Acad. Sci. USA*, **94**, 1619–1622.
51. Astatke,M., Ng,K., Grindley,N.D. and Joyce,C.M. (1998) A single side chain prevents *Escherichia coli* DNA polymerase I (Klenow fragment) from incorporating ribonucleotides. *Proc. Natl Acad. Sci. USA*, **95**, 3402–3407.
52. Cases-Gonzalez,C.E., Gutierrez-Rivas,M. and Menendez-Arias,L. (2000) Coupling ribose selection to fidelity of DNA synthesis. The role of Tyr-115 of human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.*, **275**, 19759–19767.
53. Egel,R. and Eie,B. (1987) Cell lineage asymmetry in *Schizosaccharomyces pombe*: unilateral transmission of a high-frequency state for mating-type switching in diploid pedigree. *Curr. Genet.*, **12**, 429–433.
54. Klar,A.J. (1987) Differentiated parental DNA strands confer developmental asymmetry on daughter cells in fission yeast. *Nature*, **326**, 466–470.
55. Vengrova,S. and Dalgaard,J.Z. (2004) RNase-sensitive DNA modification(s) initiates *S.pombe* mating-type switching. *Genes Dev.*, **18**, 794–804.
56. Engelke,U., Grabowski,L., Gutz,H., Heim,L. and Schmidt,H. (1987) Molecular characterization of *h*-mutants of *Schizosaccharomyces pombe*. *Curr. Genet.*, **21**, 535–540.
57. Arcangioli,B. and Klar,A.J. (1991) A novel switch-activating site (SAS1) and its cognate binding factor (SAP1) required for efficient mat1 switching in *Schizosaccharomyces pombe*. *EMBO J.*, **10**, 3025–3032.
58. Matsumoto,Y., Kim,K., Katz,D.S. and Feng,J.A. (1998) Catalytic center of DNA polymerase beta for excision of deoxyribose phosphate groups. *Biochemistry*, **37**, 6456–6464.
59. Srivastava,D.K., Berg,B.J., Prasad,R., Molina,J.T., Beard,W.A., Tomkinson,A.E. and Wilson,S.H. (1998) Mammalian abasic site base excision repair. Identification of the reaction sequence and rate-determining steps. *J. Biol. Chem.*, **273**, 21203–21209.
60. Nicholl,I.D., Nealon,K. and Kenny,M.K. (1997) Reconstitution of human base excision repair with purified proteins. *Biochemistry*, **36**, 7557–7566.
61. Callebaut,I. and Mornon,J.P. (1997) From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. *FEBS Lett.*, **400**, 25–30.
62. Garcia-Diaz,M., Dominguez,O., Lopez-Fernandez,L.A., de Lera,L.T., Saniger,M.L., Ruiz,J.F., Parraga,M., Garcia-Ortiz,M.J., Kirchhoff,T., del Mazo,J. *et al.* (2000) DNA polymerase lambda (Pol lambda),

a novel eukaryotic DNA polymerase with a potential role in meiosis. *J. Mol. Biol.*, **301**, 851–867.
63. Nick McElhinny,S.A. and Ramsden,D.A. (2004) Sibling rivalry: competition between Pol X family members in V(D)J recombination and general double strand break repair. *Immunol. Rev.*, **200**, 156–164.
64. Bebenek,K., Garcia-Diaz,M., Patishall,S.R. and Kunkel,T.A. (2005) Biochemical properties of *Saccharomyces cerevisiae* DNA polymerase IV. *J. Biol. Chem.*, **280**, 20051–20058.
65. Bonnin,A., Lazaro,J.M., Blanco,L. and Salas,M. (1999) A single tyrosine prevents insertion of ribonucleotides in the eukaryotic-type phi29 DNA polymerase. *J. Mol. Biol.*, **290**, 241–251.
66. Ferreira,M.G. and Cooper,J.P. (2004) Two modes of DNA double-strand break repair are reciprocally regulated through the fission yeast cell cycle. *Genes Dev.*, **18**, 2249–2254.
67. Karathanasis,E. and Wilson,T.E. (2002) Enhancement of *Saccharomyces cerevisiae* end-joining efficiency by cell growth stage but not by impairment of recombination. *Genetics*, **161**, 1015–1027.
68. Takata,M., Sasaki,M.S., Sonoda,E., Morrison,C., Hashimoto,M., Utsumi,H., Yamaguchi-Iwai,Y., Shinohara,A. and Takeda,S. (1998) Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO J.*, **17**, 5497–5508.
69. McCormick,P.J., Danhauser,L.L., Rustum,Y.M. and Bertram,J.S. (1983) Changes in ribo- and deoxyribonucleoside triphosphate pools within the cell cycle of a synchronized mouse fibroblast cell line. *Biochim. Biophys. Acta*, **756**, 36–40.
70. Meuth,M. (1984) The genetic consequences of nucleotide precursor pool imbalance in mammalian cells. *Mutat. Res.*, **126**, 107–112.
71. Rydberg,B. and Game,J. (2002) Excision of misincorporated ribonucleotides in DNA by RNase H (type 2) and FEN-1 in cell-free extracts. *Proc. Natl Acad. Sci. USA*, **99**, 16654–16659.
72. Kaykov,A. and Arcangioli,B. (2004) A programmed strand-specific and modified nick in *S.pombe* constitutes a novel type of chromosomal imprint. *Curr. Biol.*, **14**, 1924–1928.