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A specific loop in human DNA polymerase mu allows switching between creative and DNA-instructed synthesis

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ABSTRACT

Human DNA polymerase mu (Polμ) is a family X member that has terminal transferase activity but, in spite of a non-orthodox selection of the template information, displays its maximal catalytic efficiency in DNA-templated reactions. As terminal deoxynucleotidyl transferase (TdT), Polμ has a specific loop (loop1) that could provide this enzyme with its terminal transferase activity. When loop1 was deleted, human Polμ lacked TdT activity but improved DNA-binding and DNA template-dependent polymerization. Interestingly, when loop1 from TdT was inserted in Polμ (substituting its cognate loop1), the resulting chimaera displayed TdT activity, preferentially inserting dGTP residues, but had a strongly reduced template-dependent polymerization activity. Therefore, a specialized loop in Polμ, that could adopt alternative conformations, appears to provide this enzyme with a dual capacity: (i) template independency to create new DNA information, in which loop1 would have an active role by acting as a 'pseudotemplate'; (ii) template-dependent polymerization, in which loop1 must allow binding of the template strand. Recent *in vivo* and *in vitro* data suggest that such a dual capacity could be advantageous to resolve microhomology-mediated end-joining reactions.

INTRODUCTION

DNA template dependence is a general feature of high-fidelity DNA polymerases (most replicases and some DNA repair polymerases) in order to keep genetic information unaltered. However, some particular DNA repair mechanisms and processes oriented towards the generation of genome variability require the action of specific DNA polymerases characterized by a high error-proneness. A limit situation is when a DNA polymerase does not have any template dependence, being able to create genetic information, as it was early described for terminal deoxynucleotidyl transferase (TdT). The X family is a heterogeneous group of DNA polymerases, all of them sharing a common structural feature: a conserved Polβ core, as supported by amino acid sequence comparisons, and by extrapolation to the crystal structures of Polβ, the paradigm of the family (1), TdT (2) and Polλ (3,4). Most likely, 'template instruction' is a general feature of most members of the X family, with the exception of TdT. TdT is the only known DNA template-independent DNA polymerase, as it is able to add nucleotides to a primer DNA molecule in the absence of a template chain. This feature is crucial for its function in V(D)J recombination, where TdT adds nucleotides to the recombinational junctions of immunoglobulins and TCR receptor genes, generating variability as it creates new information (5).

One of the most recently discovered members of the X family, Polμ, is the closest homologue to TdT (sharing 42% amino acid identity), being more distant to Polβ (28% amino acid identity). Interestingly, Polμ shows hybrid biochemical properties in comparison with these other two enzymes: it has an intrinsic terminal transferase activity, but it is strongly activated by a template DNA chain (6). Moreover, a specific property of Polμ is the capacity to induce/accept template distortions, in order to realign imperfectly paired DNA primers (7). The combination of all these properties could be well suited for a role of Polμ in a non-homologous end-joining (NHEJ) DNA repair mechanism, as it was early

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proposed (7,8), and strongly supported by the demonstration of direct interactions of Polμ with NHEJ factors (9).

Important clues to understand the structural basis of the fidelity of DNA synthesis, that relies on a proper template direction, have been obtained by analysis of Polβ crystals complexed with different DNA substrates (10–14). Understanding the structural and functional basis of the 'template-independence' of TdT (and by extension for that of Polμ) had to await the resolution of the crystal structure of the Polβ-like core of TdT (2). On that basis, and by extrapolation of Polμ to the structural model of TdT, it can be predicted that an amino acid segment (loop1), specifically present in these two enzymes, could be directly responsible for their template-independent terminal transferase activity. In this paper, we show that this region of Polμ does strongly influence its template-dependent versus template-independent capacity. Moreover, the structural differences observed between Polμ and TdT suggest an important role of this region in providing Polμ with these two DNA synthesis alternatives, both of which could be beneficial for a role in NHEJ.

MATERIALS AND METHODS

Materials

Synthetic DNA oligonucleotides were obtained from Invitrogen. Desalted PCR primers used in the mutagenic PCR for generating the mutant cDNAs coding Δloop1 and Ch-loop1 variants of human Polμ were: Δ1sense d(TGTACCACCAGCACCAGCACAGCGAGAGAAGTTTCTGCATTTTCCGCC); Δ1antisense d(GGCGGAAAATGCAGAAACTTCTCTCGCTGTGCTGGTGCTGGTGGTACA); Chi1sense d(ATCCTGTACCACCAGCACCAGGAGTCAACATTTGAAAAGCTCAGGTTGCCTAGCAGGAAGGTTGATGCTTTGGATCATTTTGAGAGAAGTTTCTGCATTTTC); Chi1antisense d(GAAAATGCAGAAACTTCTCTCAAAATGATCCAAAGCATCAACCTTCCTGCTAGGCAACCTGAGCTTTTCAAATGTTGACTCCTGGTGCTGGTGGTACAGGAT). PAGE-purified oligonucleotides used to generate the DNA substrates used in biochemical assays were: P15-T d(TTTTTTTTTTTTTTT); P19 d(GATCACAGTGAGTACAATA); P15 d(TCTGTGCAGGTTCTT), P15-mis d(GATCACAGTGAGTAC); T32 d(TGAAGTCCCTCTCGACGAAGAACCTGCACAGA); T19 d(TATTGTACTCACTGTGATC); D15 d(TCGAGAGGGACTTCA). Unlabelled ultrapure dNTPs and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. T4 polinucleotide kinase and T4 DNA ligase were from New England Biolabs. TdT and Pfu DNA polymerase were from Promega. Highly purified wild-type human Polμ was obtained as described previously (6).

Amino acid sequence comparisons and 3D-modelling

A multiple alignment of different DNA polymerases of the Pol X family was done using the program MULTALIN (http://prodes.toulouse.inra.fr/multalin/) and the result was adjusted and refined manually, on the basis of the secondary structure elements of rat Polβ (11,13) and TdT (2), deduced from their respective crystal structures. Polμ tridimensional structure was modelled using the program Swiss-Model (http://swissmodel.expasy.org/SWISS-MODEL) and different 3D structures of murine TdT as a template. PDB coordinates for three different TdT structures, obtained from the Protein Data Bank (http://www.rcsb.org/pdb/), were used: 1JMS (corresponding to the crystal structure of the catalytic core of murine TdT); 1KDH [corresponding to a binary complex of murine TdT with a primer single-stranded (ss) DNA]; 1KEJ (corresponding to the crystal structure of murine TdT complexed with ddATP). As a comparison, the structure of human Polβ complexed with gapped DNA and ddCTP (1BPY) was also used. The different conformations of the modelled region corresponding to loop1 in human Polμ were analyzed by using the Swiss PDB Viewer program (http://www.expasy.ch/spdbv/). The loop1-deleted version of human Polμ (Δloop1) and and the 'chimeric' substitution of loop1 by that corresponding to human TdT (Ch-loop1) were re-modelled, and in both cases no structure alterations for the mutated proteins were predictable.

Construction and purification of mutant forms of human Polμ

Δloop1 mutant. A 17 aa deletion, corresponding to amino acid residues 369–385 (CCESPTRLAQQSHMDAF) in human Polμ, was introduced in plasmid pRSETA-hPolμ, obtained as described (6) by a PCR-based method (QuickChange® Site-Directed Mutagenesis kit, Stratagene) using Δ1sense and Δ1antisense primers whose sequence was described above. After DNA sequencing to confirm the deletion, BL21(DE3)pLysS *Escherichia coli* cells transformed with the mutant construction was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 20 min at 30°C. After 90 min of additional incubation in the presence of 120 μg/ml rifampicine, cells were collected and grounded with alumina in buffer A [20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT and 5% glycerol], supplemented with 1 M NaCl. Alumina and cell debries were pelleted by centrifugation at low speed (Sorvall GSA rotor; 5 min /3000 r.p.m.), and the cleared lysate was centrifuged for 15 min at 11 000 r.p.m. (Sorvall SS34 rotor) to separate soluble proteins from the insoluble extract (debris). The DNA present in the soluble extract was eliminated by 0.3% polyethylenimine (PEI) precipitation, and the supernatant was diluted four times with buffer A, and precipitated with ammonium sulfate to 50% saturation to obtain a PEI-free protein pellet. This precipitate, when resuspended in a non-denaturing buffer with a low ionic strength (0.1 M NaCl), rendered Δloop1 insoluble. Therefore, the pellet was resuspended in a denaturing buffer [50 mM Tris–HCl (pH 8.0), 10% glycerol, 0.5 mM EDTA, 1 mM DTT, 0.6 M NaCl and 3 M guanidinium hydrochloride], followed by a renaturation step by gentle dialysis (overnight) in buffer A, supplemented with 1 M NaCl, to allow a slow and correct refolding of the protein. After Hi-Trap Heparin (Pharmacia Biotech) chromatography of the renatured (soluble) fraction (diluted to 0.25 M NaCl with buffer A), purified Δloop1 eluted with 0.45 M NaCl. As a concentration step, the Δloop1-containing fractions were pooled and diluted to 0.1 M NaCl with buffer A, and loaded in a phosphocellulose column. Highly purified and concentrated Δloop1 was eluted in a single step with buffer A 1 M NaCl. The final fraction

(~330 ng/μl), was adjusted to 50% (v/v) glycerol, supplemented with 0.1 mg/ml BSA and stored at −70°C.

Ch-loop1. Amino acid residues 381 to 400 (ESTFEKLRLPSRKVDALDHF) of human TdT were inserted in plasmid pRSETA-hPolμ (6), instead of amino acid residues 367–385 (HSCCESPTRLAQQSHMDAF) of human Polμ. Thus, a chimeric construct of human Polμ, whose putative loop1 has been substituted by that from human TdT, was obtained. The construct was obtained by mutagenic PCR using oligonucleotides Chi1sense and Chi1antisense. After DNA sequencing to confirm the chimeric construct, BL21(DE3)pLysS *E.coli* cells transformed with the mutant construction were induced with 0.5 mM IPTG for 20 min at 30°C. The overproduced Ch-loop1 protein remained soluble and was purified essentially as described for the wild-type enzyme (6).

DNA polymerization substrates

DNA oligonucleotides used as primers (P19, P15 and P15-mis, P15-T) were labelled at its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Some oligonucleotides primers (P) were hybridized to one template DNA oligonucleotide (T), or simultaneously to both template (T) and downstream (D) DNA oligonucleotides, in the presence of 50 mM Tris–HCl (pH 7.5) and 0.3 M NaCl to generate the following DNA substrates: blunt-ended DNA (P19 + T19), as these oligonucleotides are fully complementary; template/primer (P15 + T34), having a 5′ extension of 19 template nucleotides; gap2-P (P15 + T32 + D15), forming a gap of two template nucleotides flanked by a 5′-phosphate group; and mismatched primer/template (P15-mis and T22), in which only the 3′-terminal base of the primer is not properly paired to the template strand.

Electrophoretic mobility shift assay (EMSA) analysis

To analyze Polμ/DNA interactions, different 5′ P-labelled DNA polymerization substrates (4 nM) were incubated with various concentrations of the indicated protein for 10 min at 30°C, in 12.5 μl of 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 4% glycerol and 0.1 mg/ml BSA, and then, the incubation mixture was adjusted to 10% glycerol and loaded in 4% native polyacrylamide gels in 5× TAE and 50 mM EDTA (pH 7.5). After electrophoresis at 180 mA/4°C, the protein–DNA complexes, producing a shift in the position of the labelled free DNA were detected by autoradiography. When indicated, DNA-binding affinity was estimated from the reduction of the input DNA substrate running as free DNA produced by different dosis of DNA polymerase, determined by densitometric scanning of the autoradiographs.

DNA polymerization assays

To analyze DNA-dependent DNA polymerization, different DNA substrates, containing 5′ P-labelled primers (described above) were incubated with either the wild-type human Polμ or its Δloop1 deletion derivative, at the concentration indicated in each case. The reaction mixture, in 12.5 μl, contained 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 4% glycerol and 0.1 mg/ml BSA, in the presence of 4 nM of the indicated DNA polymerization substrate, and the indicated concentration of dNTP and activating metal ions. After incubation for 30 min at 30°C, reactions were stopped by adding gel loading buffer [95% (v/v) formamide, 10 mM EDTA, 0,1% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue] and analyzed by 8 M urea/20% PAGE and autoradiography. To analyze TdT activity, a homopolymeric 5′ P-labelled single-stranded oligonucleotide (P15-T), made of 15 T residues, was used to avoid the formation of secondary structures that could mimic a template/primer situation. When indicated, a blunt-ended DNA substrate (P19/T19) was also used. The reaction mixture, in 12.5 μl, contained 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 4% glycerol and 0.1 mg/ml BSA, 4 nM of either 5′ P-labelled P15-T or P19/T19, the indicated amount of DNA polymerase (either TdT, or the wild-type human Polμ, or mutant Δloop1, or the Ch-loop1 chimera), and the indicated concentration of dNTP and activating metal ions. After incubation for 30 min at 30°C, reactions were stopped by adding gel loading buffer [95% (v/v) formamide, 10 mM EDTA, 0,1% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue] and analyzed by 8 M urea/20% PAGE and autoradiography.

Steady-state kinetics analysis

For this analysis, incorporation of dGTP was measured on T22/P15-mis, a DNA substrate that can realign the mismatched primer-terminus using a neighbour complementary templating base, thus allowing dGTP selection and insertion to occur as a template-directed event. The reaction mixture, in 12.5 μl, contained 50 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 4% glycerol and 0.1 mg/ml BSA and 200 nM T22/P15-mis. To set up steady-state conditions, a saturating concentration of dGTP (100 μM) was added, and various concentrations of protein (either Polμ wt or Δloop1) and reaction times were assayed to obtain an amount of extended primers not higher than 30% of the total input. Under the conditions selected (5 nM enzyme /15 min reaction), reaction velocity (fmol/min) was determined as a function of dGTP concentration (from 0.1 to 100 μM). The plotted data were fitted by a non-linear regression curve to the Michaelis–Menten equation:

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

Using Kaleida Graph software (Synergy Software, www.synergy.com) V_{max} and K_m values were obtained from the fitted curves, and the catalytic constant (K_{cat}) and Catalytic Efficiency (Cat Eff) values were calculated from the following equations:

$$K_{cat} = V_{max}/[\text{enzyme}] \quad \mathbf{2}$$

$$\text{Cat Eff} = K_{cat}/K_m \quad \mathbf{3}$$

RESULTS

A structural loop potentially involved in the terminal transferase activity of Polμ

By comparison of the 3D structures of Polβ and TdT, a TdT-specific loop (loop1; 383–400 amino acids), located in the palm subdomain (see Figure 1A), was proposed to be important for the specific properties of TdT (2). By inspection of the multiple amino acid sequence alignments of Polβ, TdT and

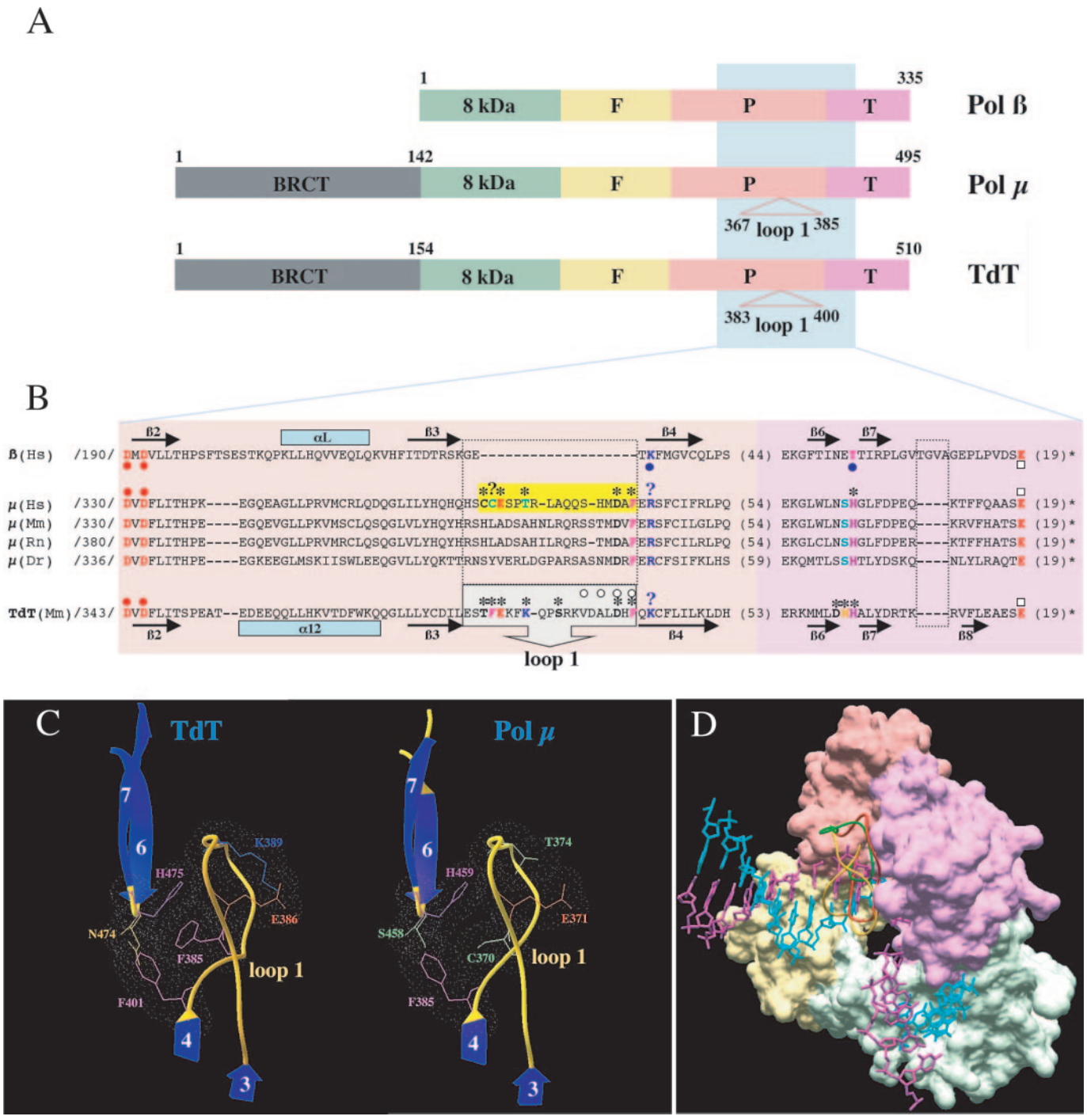

Figure 1. TdT and Polμ have a specific loop at the palm subdomain. (**A**) Scheme of the multidomain structure of Polβ, Polμ and TdT. The N-terminal region containing a BRCT domain is only present in Polμ and TdT. Other domains, as 8 kDa, fingers (F), palm (P) and thumb (T) are shared by the three enzymes, and constitute an evolutionarily conserved Polβ polymerization core, present in most members of the DNA polymerase family X. The insert shows the position and flanking residues corresponding to loop1, exclusively present in the palm subdomain of TdT and Polμ. (**B**) Amino acid sequence alignment of human Polβ, mouse TdT and Polμ from different species (Hs, human; Mm, mouse; Rn, rat; Dr, trout) along the region containing loop1 in Polμ and TdT. Invariant residues acting as metal ligands are indicated with red dots. Polβ residues acting as DNA ligands are indicated with blue dots. Asterisks indicate putative residues involved in interactions of loop1 with the thumb subdomain. The region deleted in Δloop1 mutant is boxed in yellow. (**C**) Prediction of a mobile loop1 in Polμ as a switcher between creative and DNA-directed DNA synthesis. Some of the amino acid residues in TdT that are involved in the stabilization of loop1 (via connection to the thumb) are not conserved in Polμ. Particularly, the strong stacking interaction between the aromatic rings of TdT residues Phe385 (loop1) and His475 (thumb), is not conserved in Polμ (substituted by a cysteine (Cys370) residue). (**D**) By modelling human Polμ on the crystal structure of TdT (either as apoenzyme (PDB id: 1JMS), or complexed with dNTP(PDB id: 1KEJ) or ssDNA (PDB id: 1KDH), alternative conformations of loop1 can be predicted for Polμ. In this figure, represented by using the Swiss PDB Viewer program (http://www.expasy.ch/spdbv/), a gapped DNA has been also modelled based on a Polβ ternary complex (PDB id: 1BPY). Template strand is in magenta; the different conformations of loop1 are shown in red, yellow and green colours; subdomains in Polμ are light coloured in green (8 kDa), yellow (fingers), red (palm) and magenta (thumb), as shown in (A).

Polμ (2,6,8) it is apparent that a homologous region to loop1 is present also in Polμ (367–385 amino acids; see Figure 1A and B). Moreover, tridimensional modelling of Polμ on the TdT crystal structure, carried out as described in Materials and Methods, predicts a similar spatial location of the region homologous to loop1, located in the palm subdomain (data not shown).

As template-independent polymerization is a specific property of TdT, which is also present in Polμ (6), it can be argued that the terminal transferase activity could be somehow related to this loop. To test this hypothesis, a deletion derivative of human Polμ lacking the amino acid segment corresponding to residues 369–385 (17 amino acid), and therefore lacking most of loop1, was constructed. Only two residues (His367 and Ser368) corresponding to loop1 in human Polμ were maintained in the truncated form, as they could be important for a Polβ-like connection between the flanking beta strands. This truncated form of human Polμ (Δloop1) was overproduced in *E.coli* and purified to homogeneity, as indicated in Materials and Methods.

Deletion of loop1 in Polμ improves binding to DNA substrates having a single-stranded template chain

As Polμ is preferentially a DNA-dependent DNA polymerase, although having some TdT-like activity, it was important to evaluate the DNA binding preferences of this enzyme that could be related to its dual polymerization capacity. By using gel-shifting experiments, different DNA substrates mimicking DNA repair intermediates were analyzed for their capacity to form stable complexes with human Polμ. As shown in Figure 2, human Polμ did not produce stable complexes either with ssDNA or with double-stranded/blunt-ended DNA molecules (Blunt), in agreement with its weak TdT activity. Conversely, stable enzyme–DNA complexes could be readily seen when using DNA molecules providing a ssDNA portion suitable as a templating strand (either forming a gapped-substrate or not). Therefore, this preference to bind DNA template-containing molecules is in agreement with the definition of human Polμ as a DNA-dependent DNA polymerase (6), irrespective of its strong tendency to induce and/or accept template/primer misalignments (7). In fact, the different complexes (bands 'a' and 'b' in Figure 2) observed in the EMSA assays suggest the existence of alternative binary complexes that could be related to the capacity of Polμ to induce misalignments.

At this point, it was important to test if loop1 had some influence in the substrate preference and DNA-binding capacity of human Polμ. Thus, when Δloop1 mutant was assayed under the same conditions as Polμ wt, it showed the same substrate preferences, giving rise to stable enzyme–DNA complexes only with substrates providing an available template chain. However, as shown in Figure 3, quantitation of the DNA binding capacity of wild-type versus Δloop1 mutant Polμ indicated a large gain (~3 to 4-fold) in DNA affinity displayed by the deleted form. A similar result was obtained using either template/primer (Figure 3A) or gapped (Figure 3B) DNA molecules. These results suggest that loop1 is dispensable for DNA template binding, and its presence negatively affects the maximal DNA binding capacity of Polμ.

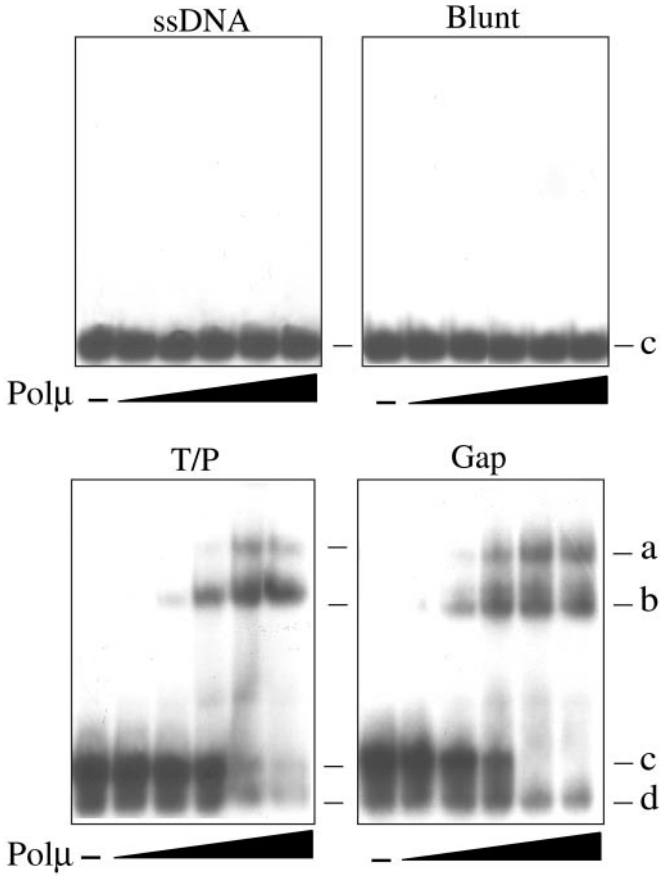

Figure 2. DNA-binding preferences of human Polμ. Formation of stable complexes between the wild-type Polμ and DNA was comparatively studied using the following DNA molecules, corresponding to different DNA repair intermediates: ssDNA, blunt-ended DNA (Blunt), template/primer (T/P) and 2 nt-gapped DNA (Gap). The assay was carried out using 4 nM of the corresponding labelled DNA primer, either alone or pre-hybridized to a DNA template (and to a downstream oligonucleotide, in the case of gap2-P), as described in Materials and Methods. Increasing concentrations (36, 72, 144, 432 and 864 nM) of Polμ wt were added to the reaction [50 mM Tris–HCl (pH 7.5), 1 mM DTT, 4% glycerol and 0.1 mg/ml BSA] and incubated 10 min at 30°C. Enzyme–DNA complexes (a and b) were identified by their retarded migration after 4% native PAGE and autoradiography. c, free hybridized DNA substrate; d, rest of non-hybridized primer.

Loop1 of Polμ is not required for template dislocation and primer realignment

The most peculiar feature of Polμ resides in its strong capacity to accept/induce template distortions, leading to misincorporation. Such an irregular usage of the template strand is not only displayed on template/primer molecules with long template strands (7), but also in DNA with small gaps as short as 1 nt, in which template dislocation can occur either mediated by slippage or by incoming dNTP selection (15). To determine if loop1 is involved in template dislocation, Polμ wt and Δloop1 were first compared with their capacity to produce misincorporation on a 2 nt-gapped DNA substrate (Figure 4A). The ratio of primers extended with dCTP versus those extended with dGTP (about 0.25) indicates that wild-type Polμ has 4-fold preference for using the second templating nucleotide (dC) to direct the first addition to the primer (dG). In this case, the template dislocation required

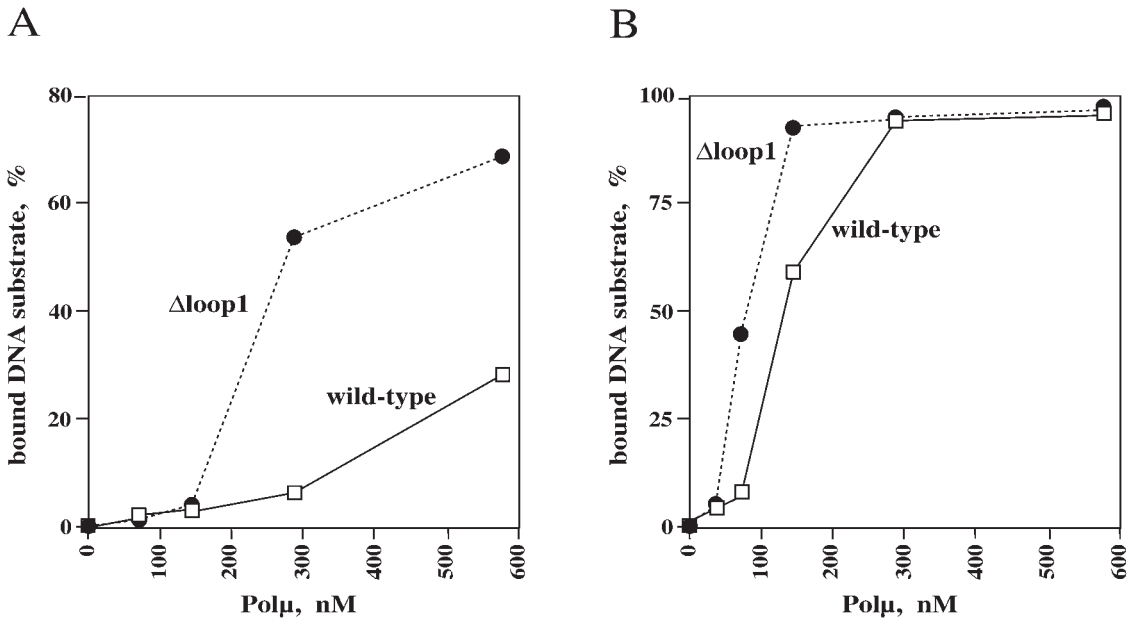

Figure 3. Deletion of loop1 improves binding of Polμ to templated-DNA molecules. As shown in the figure for either a template/primer (**A**) or a 2 nt-gapped DNA substrate (**B**), elimination of loop1 in Polμ improves its DNA binding capacity (assessed by gel-shifting experiments as described in Figure 2), suggesting that loop1 would have some negative effect on DNA-directed DNA synthesis, probably interfering with the template binding region of human Polμ.

(unpairing a G residue) is stabilized by the incoming (dGTP) nucleotide. As shown in Figure 4A, mutant Δloop1 extended the primer with a similar preference for dGTP versus dCTP as Polμ wild-type. Such a template dislocation capacity provides a basis to extend mismatched primers or to stabilize microhomology-mediated joining of broken DNA ends (7). An example of the former reaction is shown in Figure 4B, in which Polμ extends the mismatched primer-terminus (dC), by inducing its pairing (via realignment) to a close complementary base (dG) of the template strand, and a further insertion of the next complementary nucleotide (dGTP). As shown in Figure 4B, mutant Δloop1 was even more efficient than the wild-type to realign and extend the mismatched primer-terminus. So, it can be concluded that the lack of loop1 did not alter the mistemplating and primer realignment capacity characteristic of Polμ. These assays were carried out at 600 nM of enzyme, a concentration high enough to minimize DNA binding differences between Polμ wt and Δloop1. Therefore, the fact that both dCTP and dGTP insertion were more efficiently catalyzed by Δloop1 mutant in comparison to the wild-type enzyme, suggests that elimination of loop1 could also modify the kinetic parameters of the polymerase.

Loop1 deletion improves catalytic efficiency in DNA-templated reactions

As shown in the previous experiments, elimination of loop1 did not change the substrate preferences and specific properties of Polμ, but produced a remarkable improvement in DNA binding and catalysis. To verify this point, analysis of the kinetic parameters under steady-state conditions was carried out. A DNA molecule with a mismatched primer-terminus (as that shown in Figure 4B), susceptible to be realigned to a neighbouring complementary template base, was selected as the substrate. After fitting the data to the Michaelis–Menten equation, apparent V_{max} and K_m values were calculated (see Table 1). An increase of ~5.5-fold in the catalytic constant (k_{cat}) together with a 2.3-fold K_m reduction for mutant Δloop1 versus the wild-type, indicates that elimination of loop1 produces a 12.9-fold improvement of the catalytic efficiency in this particular DNA-templated reaction.

Loop1 is required for DNA-independent DNA polymerization

The experiments described above demonstrated that loop1 is not required either for binding a template-containing DNA substrate, or for DNA-dependent DNA polymerization itself. Moreover, elimination of loop1 did not affect the peculiar capacity of Polμ to induce template/primer distortions and realignments. In fact, elimination of loop1 had positive consequences for template-dependent DNA polymerization, since both DNA binding and catalytic efficiency turned out to be improved. So, at this point, it was important to test if loop1 was necessary for the catalysis of DNA-independent DNA polymerization reactions, i.e. TdT-like reactions. To discard the possibility that a heteropolymeric ssDNA substrate can form either intramolecular or intermolecular base pairing that can provide 'pseudo templated' primer-termini, a Poly(dT) homopolymer (15mer) was selected as primer to test TdT activity.

As shown in Figure 5A, TdT was able to extend this untemplated primer when provided with any of the four dNTPs, but there are differences in the elongation pattern depending on the dNTP used in each case. Thus, TdT showed a marked preference to generate long products (>20 nt) by reiterative addition of dGMP units. dCTP was also efficiently used, but generating smaller elongation products. On the

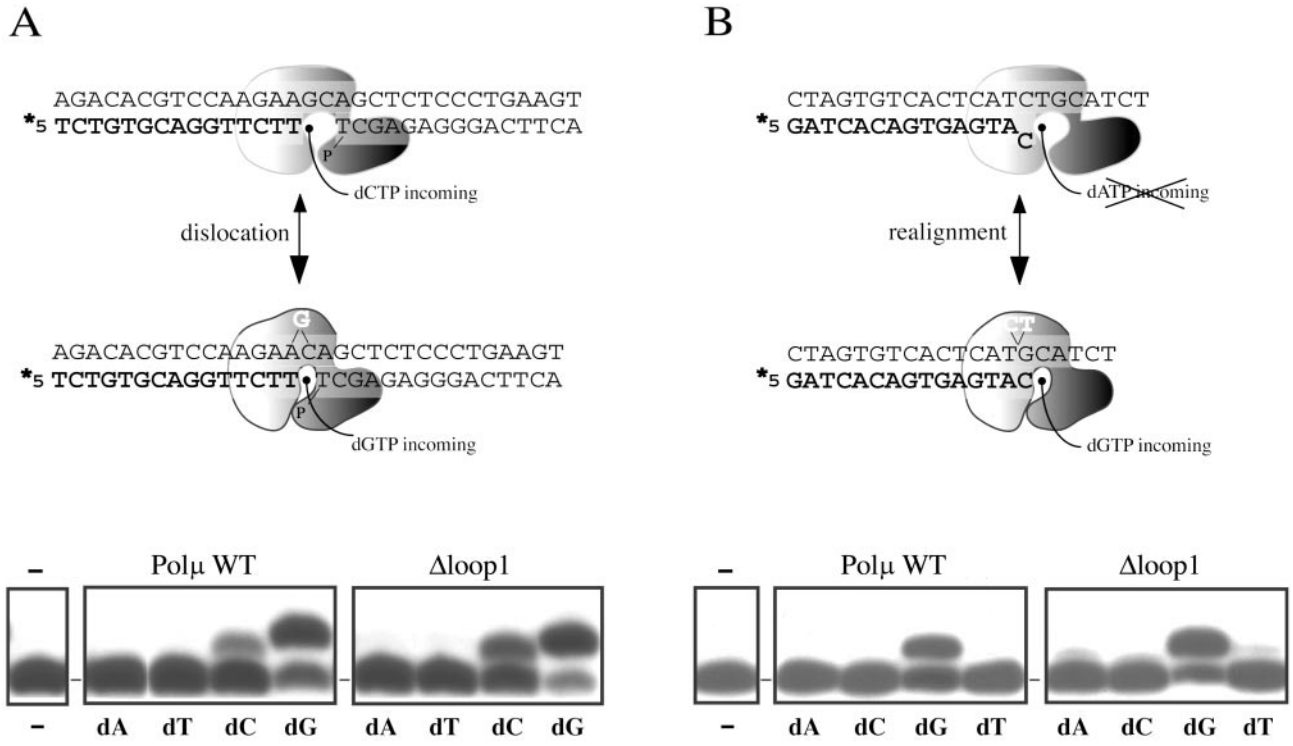

Figure 4. Deletion of loop1 does not affect template misalignment-mediated misincorporation and primer realignment by Polμ. (**A**) On a 2 nt-gapped DNA substrate (4 nM), deletion of loop1 did not destroy the capacity of human Polμ to catalyze a preferred insertion of dG in front of the second template base of the gap (dC). This 'apparent misinsertion' occurs as a consequence of Polμ's proneness to produce misalignement of the template dG, stabilized by the incoming dGTP nucleotide ('dNTP selection mechanism'). (**B**) Loop1 is irrelevant to Polμ in order to realign a mismatched terminus to a distant complementary position, as it was described for the wild-type human Polμ (7). Insertion of dGTP in front of a distant dC template base is thus observed in both wild-type and Δloop1 mutant. In both cases (A and B) the reaction was carried out in the presence of 2 mM $MgCl_2$,1 μM of the indicated dNTP, and 600 nM of either Polμ wt or Δloop1 as indicated. After incubation for 30 min at 30°C, the extension of the 5′ P-labelled oligonucleotide was analysed by 8 M urea and 20% PAGE and autoradiography.

Table 1. Loop1 deletion improves the catalytic efficiency of Polμ when catalyzing a templated insertion

	K_{cat} (s^{-1})	K_m (app.) μM	Catalytic efficiency $\mu M^{-1}s^{-1}$
Δloop1			
Assay 1	0.00973	3.078	0.00316
Assay 2	0.0116	4.140	0.00280
Assay 3	0.0100	4.739	0.00212
Average ± SD	0.0104 ± 0.00101	3.986 ± 0.8412	0.00269 ± 0.0005282
Wt			
Assay 1	0.00164	10.487	0.000157
Assay 2	0.00219	10.718	0.000205
Assay 3	0.00184	6.900	0.000266
Average ± SD	0.00189 ± 0.0002274	9.3385 ± 1.749	0.0002085 ± 0.0000446

The table compares the kinetic parameters of polymerization of Polμ wt versus Δloop1 when assayed on a DNA substrate susceptible of template dislocation. Figures indicate the values of K_{cat}, K_m and catalytic efficiency, obtained from various independent experiments (described in Materials and Methods). The mean values (average) and standard deviation are indicated.

other hand, TdT was very inefficient with dTTP and dATP on this Poly(dT) substrate, only incorporating a few nucleotides. Comparatively, human Polμ gave a more homogeneous pattern of extension with any of the four dNTPs. Exceptionally, addition of dATP produced some amount of a longer product, whose size (~25 nt) suggests that after DNA-independent addition of some dAMP units to the primer, the latter can be paired (end-joined) to a second Poly(dT) molecule that can now act as a template for further dAMP insertion. In agreement with that, such a product was absent when using Poly(dA) as primer and dATP, but was present again when using Poly(dA) and dTTP (data not shown). As a control, using these homopolymeric ssDNA substrates, human Polλ (that lacks an equivalent loop 1), was inactive (data not shown).

Interestingly, as shown in Figure 5A, mutant Δloop1 did not extend the primer Poly(dT) with any of the four dNTPs, indicating that loop1 is critical for such a

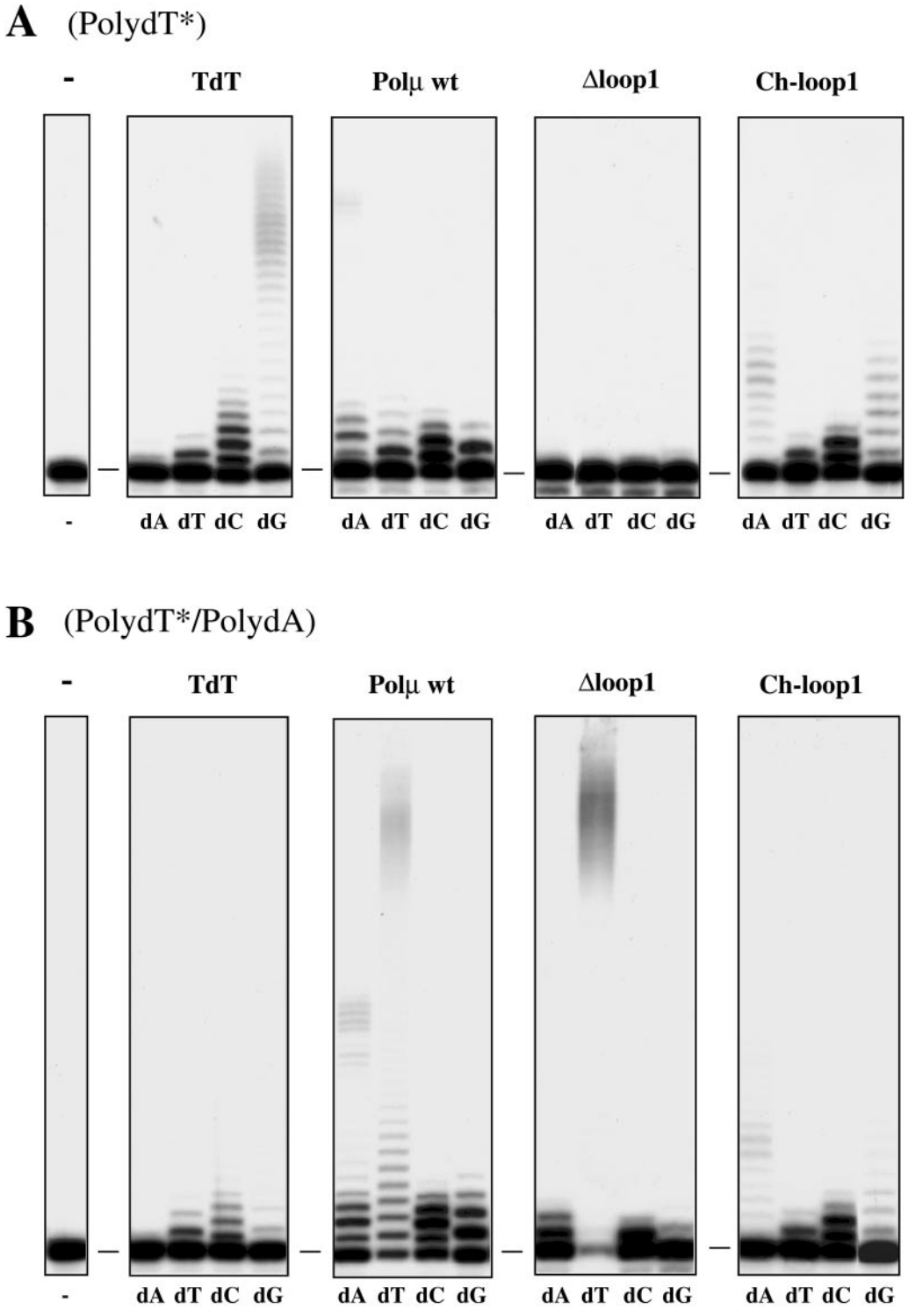

Figure 5. Loop 1 is critical for terminal transferase activity. (**A**) The assay was carried out using 4 nM of a labelled homopolymer (P15-T) as DNA primer. The reaction, carried out as described in Materials and Methods, was carried out in the presence of 1 mM $MnCl_2$, 10 μM of the indicated dNTP, and 600 nM of either Polμ wt, or Δloop1, or Ch-loop1, or commercial TdT (3 U). After incubation for 30 min at 30°C, the extension of the 5′-labelled oligonucleotide was analysed by 8 M urea and 20% PAGE and autoradiography. (**B**) The assay was identical to that described in part A, but providing Poly(dT)*/ PolidA as a template.

template-independent DNA polymerization reaction. The same result was obtained with different DNA substrates, as Poly(dA) or blunt-ended DNA molecules (data not shown). Such a reduction in TdT activity contrasts with the improvement of DNA-dependent DNA polymerization activity when loop1 is eliminated, suggesting that loop1 can regulate the switch/balance between DNA-independent and DNA-dependent polymerization by Polμ.

A chimeric construct in which loop1 from TdT was inserted in Polμ displays terminal transferase activity, but a largely reduced DNA-dependent polymerization

Since loop1 appeared to be essential for TdT activity, we wanted to check if loop1 from human TdT and human Polμ are interchangeable. So, based in the 3D-structural information available for TdT, and in amino acid sequence comparisons (discussed later in Figure 1B), a chimeric construct (Ch-loop1) in which amino acid residues 367–385 of human Polμ were eliminated, being substituted by the corresponding residues (381–400) from human TdT, was obtained as described in Materials and Methods.

Interestingly, Ch-loop1 displayed TdT activity, but showed an altered pattern in comparison with the wild-type human Polμ. As shown in Figure 5A, Ch-loop1 produced a ladder of extension products when purine deoxynucleotides (either dATP or dGTP) were provided, whereas a more limited extension was obtained with pyrimidine deoxynucleotides (either dT or dC). Overall, Ch-loop1 displays a hybrid phenotype between TdT and Polμ, mimicking the extension pattern of TdT with dT and dG, but behaving like Polμ when dC and dA are provided. Moreover, the Ch-loop1 mutant did not produce the long product obtained with Polμ in the presence of dATP, suggesting that insertion of loop1 from TdT is affecting DNA-dependent DNA polymerization by Polμ. To clarify this point, we carried out the same reactions shown in Figure 5A, but providing a complementary strand [Poly(dA)] to the labelled Poly(dT) primer. As shown in Figure 5B, TdT showed a slightly reduced efficiency, but a similar pattern of usage of dATP, dTTP and dCTP. Interestingly, the insertion of the preferred nucleotide (dGTP) was strongly inhibited, and therefore not as a direct consequence of competing amounts of 3′-termini [those corresponding to the unlabelled Poly(dA) molecule] but to the apparent inability of TdT to extend a dA:dTMP primer-terminus. Conversely, in the presence of Poly(dA), Poly(dT) primers were more efficiently extended by human Polμ (compare Figure 5A and B), in agreement with its preference to bind template/primer versus ssDNA molecules (see Figure 2). By providing either dCTP, dATP or dGTP [which are non-complementary to the Poly(dA) template], the reaction observed mainly reflects the terminal transferase capacity of human Polμ, which is not inhibited but slightly stimulated by the addition of Poly(dA). However, addition of dTTP, which is complementary to the Poly(dA) template, produced the most efficient extension of the Poly(dT) primer, reflecting the higher efficiency of Polμ in DNA-templated polymerization reactions. In an agreement with the data shown in Figure 5A, mutant Δloop1 displayed a very reduced TdT activity (tested with either dA, dC or dG), on a Poly(dT)/Poly(dA) hybrid (see Figure 5B). However, and also in agreement with its improved catalytic efficiency for DNA-templated polymerization, there was no inhibition but stimulation, when dTTP was provided (Figure 5B). Strikingly, the chimeric construct Ch-loop1 was very inefficient to catalyze dTTP insertion on the Poly(dT)/Poly(dA) substrate in comparison with both the wild-type Polμ and Δloop1 mutant, mimicking again the behaviour and substrate preference of human TdT.

In conclusion, loop1 is required for TdT activity, and interferes with DNA-dependent DNA polymerization. Such an interference can be maximized by substituting loop1 in Polμ with the corresponding loop1 from TdT.

DISCUSSION

An obvious feature relating Polλ, Polμ and TdT (but absent in Polβ) is the presence of an N-terminal BRCT domain (16), used by the three Pols to interact with NHEJ/V(D)J recombination factors (17,18). However, identification of less obvious structural differences requires a detailed inspection of the available X-ray structures of Polβ, Polλ and TdT cores. As a result, the importance of a specific amino acid segment (loop1), inserted in the palm subdomain of Polμ and TdT, could be anticipated (2).

A human Polμ mutant, lacking a segment of 17 amino acids predicted to form loop1, was constructed, overexpressed in *E.coli* cells, and purified to homogeneity. As expected from its presence in TdT, loop1 was not required for template-directed DNA polymerization. Moreover, the lack of loop1 did not eliminate the capacity of Polμ to accept/induce template misalignments during polymerization. In fact, elimination of loop1 positively affected the interaction of Polμ with a templated-DNA substrate, and improved the kinetic parameters corresponding to a template-directed DNA polymerization reaction. Therefore, and in agreement with TdT structural data (2) and our modelling studies with Polμ, loop1 appears to act as a steric hindrance for binding the template strand of a DNA substrate. Strikingly, Δloop1, although having an enhanced templated polymerization capacity, was devoid of terminal transferase activity, unambiguously assayed on single-stranded homopolymeric substrates and with addition of non-complementary dNTPs. Therefore, as it can be predicted also in the case of TdT, loop1 appears to be essential for template-independent nucleotide addition. Based on the analysis of the crystal structure of TdT complexed with ssDNA (PDB id: 1KDH) and with ddNTP (PDB id: 1KEJ), this loop would substitute the function of a template strand, providing stability (not selection) to an incoming nucleotide, thus allowing terminal transferase activity.

A mobile loop1 in Polμ could allow both template-dependent and template-independent DNA polymerization

As shown in Figure 1B, a detailed comparison of the amino acid sequence corresponding to loop1 in both Polμ and TdT allowed to identify some conserved residues, as those stabilizing the loop itself (Thr384 and Asp399 in TdT; Cys369 and Asp383 in Polμ), but also some important differences in the connectivity of loop1 (located inside the palm) with its respective thumb subdomain. Interestingly, TdT residue Phe385 of loop1 (invariant in all TdTs) interacts with residue His475 in the thumb, via a planar interaction between their aromatic rings (see Figure 1C). Moreover, His475 is hydrogen-bonded to the carbonyl atom of Lys394 of loop1 (2). Additionally, another aromatic TdT loop1 residue, Phe401, interacts with Asn474 of the thumb. Finally, a water molecule bridges the hydroxyl group of Ser392 and the side chain of Asp473 (2). These interactions probably

maintain the structure of TdT loop1 connected with the thumb in a kind of loop1 'closed' mode. Conversely, Polμ lacks some of these residues, having a small residue (Cys370) at the equivalent position of Phe385 in TdT (see Figure 1B and C). The other connecting residue of the loop1 is conserved (Phe385), but it would establish a looser interaction with Ser458 at the thumb. Therefore, this different connectivity could imply the existence of alternative configurations of the Polμ loop1, as those modelled in Figure 1D. In fact, our mutational analysis indicates that the loop1 in Polμ can adopt an 'opened' mode, in which a template strand can occupy the regular position to direct dNTP selection. However, although connectivity is predicted to be much weaker, the fact that Polμ has some capacity to drive terminal transferase at the end of ssDNA indicates that its loop1 appears to have some capacity to mimick TdT's 'closed' mode.

To further support our hypothesis, we made a construct in which an amino acid segment containing loop1 in TdT (20 amino acids) was inserted in Polμ instead of its corresponding loop1 (19 amino acids). The purified Polμ-TdT chimera (Ch-loop1) behaved exactly as expected: (i) terminal transferase activity was proficient, although its specificity, in terms of dNTPs preference, was intermediate between that of Polμ and TdT; (ii) DNA-dependent DNA polymerization was strongly inhibited. Therefore, it appears that the resulting chimera has a less mobile loop1, probably due to the interaction between the Phe residue that substitutes Polμ Cys370 of the loop and residue His459 (Polμ) in the thumb, precluding template binding and subsequent nucleotide selectivity. Thus, the more rigid configuration of the chimeric loop alters Polμ's balance of template dependence, which is now commited to perform terminal transferase-like nucleotide additions.

It has been shown that Polμ efficiently extends a primer located in front of an abasic site (19). Interestingly, this extension occurs either via primer realignment if the sequence context is appropriate or, alternatively, via a mode of nucleotidyl transferase activity, which does not depend on the sequence of the template. In the latter case, the lack of a sole templating base could be enough to activate loop1, entering in a 'closed' mode that would allow a tight interaction with any incoming nucleotide. Based on these results, it was proposed that Polμ is a dual mode DNA-synthesizing enzyme, which can act as either a classical DNA polymerase or as a non-canonical, template-dependent, but sequence-independent nucleotidyl transferase, and that these activities may be required for its function in NHEJ in the processing of DNA ends prior to ligation (19). Following up this hypothesis, our results would support an important role of loop1 in the NHEJ function of Polμ.

How important is loop 1 *in vivo*?

In contrast to other defficiencies in NHEJ proteins, single KO models of DNA polymerases Polμ and Polλ are viable, leading to initial suggestions that they were partially redundant (20–22). However, Polμ is essential to carry out accurate immunoglobulin light chain rearrangement, and its defficency originates a severe reduction of B cells in peripheral lymphoid organs due to increased deletion during NHEJ (22,23). As it has been recently shown (18), loop1 is dispensable to interact with the NHEJ factors Ku and the XRCC4-ligase IV complex to form a stable complex on DNA *in vitro,* and to promote NHEJ *in vitro* of DNA ends with microhomology; conversely, loop1 is indispensable for Polμ's unique ability to synthesize across ends aligned with no homology, as it occurs during immunoglobulin light chain rearrangement. Therefore, this loop1-dependent unique activity of Polμ is sufficient to explain the deficiency in light chain rearrangement seen in Polμ KO mice (23). A possibility is that loop1 requirement is related to Polμ's TdT-like ability to act on ends with 3′ overhang primers (as shown in Figure 5A). However, unlike TdT, activity of Polμ during light chain recombination is not typically associated with frequent N-additions (20,23). We consider two possible explanations for how the role of these two polymerases in NHEJ diverge. As previously suggested, differences in loop1 between Polμ and TdT may allow Polμ to better accommodate template at the site of synthesis within the NHEJ complex, allowing Polμ to perform template-dependent synthesis (18). Alternatively, Polμ may initially perform template-independent synthesis, much as TdT does. The occurrence of these additions could be inadverted/masked because the increased mobility of Polμ's loop1 relative to TdT would allow annealing of fortuitous microhomologies within these random additions, and Polμ would thus synthesize across non-complementary DNA ends in a manner that only appears to be template-dependent.

CONCLUSION

In 2000, human Polμ was shown to have a weak TdT activity that, under optimal conditions, was 370-fold lower than its template-dependent polymerase activity (6). As shown here, this TdT-like activity requires the presence of a specific segment, originally designated as loop1 according to TdT structure (2). It is very likely that stabilization of loop1 at specific template strand contexts as an abasic site or NHEJ intermediates, can enhance this dormant TdT activity present in Polμ. Thus, whereas loop1 in TdT is committed to allow template-independent synthesis as the unique possibility, based on steric incompatibility with a neighbour piece of template strand, Polμ could use its different (probably more mobile) loop1 under two different circumstances: (i) to stabilize a 'free', non-complementary, template end, thus putting templating bases in register at the active site. (ii) In the complete absence of a template strand (perhaps as an initial step in some NHEJ contexts), loop1 would trigger TdT activity (N-additions) widening the repertoire of end-joining solutions. If N-additions can be used to bridge primer and template as a first 'joining event', they would compensate for the frequent deletions associated with NHEJ, thus reducing the loss of genetic material. A more detailed view of the structure/function mechanics of loop1 must await for the resolution of the X-ray structure of Polμ complexed with non-complementary NHEJ intermediates.

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