The EMBO Journal Vol.19 No.7 pp.1731–1742, 2000

DNA polymerase mu (Pol μ), homologous to TdT, could act as a DNA mutator in eukaryotic cells

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This work is dedicated to the memory of Professor Eladio Viñuela

A novel DNA polymerase has been identified in human cells. Human DNA polymerase mu (Pol μ), consisting of 494 amino acids, has 41% identity to terminal deoxynucleotidyltransferase (TdT). Human Pol μ, overproduced in *Escherichia coli* in a soluble form and purified to homogeneity, displays intrinsic terminal deoxynucleotidyltransferase activity and a strong preference for activating Mn^{2+} ions. Interestingly, unlike TdT, the catalytic efficiency of polymerization carried out by Pol μ was enhanced by the presence of a template strand. Using activating Mg^{2+} ions, template-enhanced polymerization was also template-directed, leading to the preferred insertion of complementary nucleotides, although with low discrimination values. In the presence of Mn^{2+} ions, template-enhanced polymerization produced a random insertion of nucleotides. Northern-blotting and *in situ* analysis showed a preferential expression of Pol μ mRNA in peripheral lymphoid tissues. Moreover, a large proportion of the human expressed sequence tags corresponding to Pol μ, present in the databases, derived from germinal center B cells. Therefore, Pol μ is a good candidate to be the mutator polymerase responsible for somatic hypermutation of immunoglobulin genes.

Keywords: human DNA polymerase/mutator/somatic hypermutation/terminal deoxynucleotidyltransferase

Introduction

The maintenance and stability of genetic information in DNA depends largely on the high fidelity of DNA synthesis displayed by replicative polymerases, which in most cases are capable of proofreading insertion errors (reviewed by Bebenek and Kunkel, 1995). A further improvement in DNA stability relies on different systems of DNA repair, able to detect and repair most kinds of DNA damage that, if left unrepaired, could lead to cell transformation and death. Despite all this DNA maintenance enzymology, capable of excising bases and nucleotides, repairing breaks and correcting mismatches, there is some 'necessary risk' of accumulating DNA mutations as the driving force allowing evolution. Such background mutability could be due to misfunction of replication and the repair machinery, but also to the participation of a specific enzymology, contributed by mutator (error-prone) DNA polymerases, which should work in opposition to DNA repair.

DNA polymerase beta (Pol β) is one of the known cellular DNA polymerases for which a specific role in DNA repair has been proposed (Wilson, 1998; Dianov *et al*., 1999). However, a working hypothesis is that some altered versions of Pol β could represent mutator DNA polymerases acting as dominant error-prone enzymes capable of altering the normal levels of DNA repair (Bhattacharyya and Banerjee, 1997; Clairmont and Sweasy, 1998; Clairmont *et al.*, 1999). Recently, three novel non-essential cellular DNA polymerases, zeta (ζ; Lawrence and Hinkle, 1996), eta (η; Johnson *et al*., 1999a) and theta (θ; Sharief *et al*., 1999) have been reported. Pol ζ and Pol η are capable of altering the outcome of the DNA repair process, since these enzymes are able to use damaged (unrepaired) DNA efficiently as a template (see Friedberg and Gerlach, 1999). In yeast and fungi, most spontaneous and damage-induced mutations are introduced by Pol ζ responsible for trans-lesion DNA synthesis (Han *et al*., 1998). A model for somatic hypermutation of Ig genes has been proposed whereby Pol ζ is recruited to the Ig locus (Diaz and Flajnik, 1998). Pol η is required for error-free replication of UV lesions, and its absence produces the variant (V) form of xeroderma pigmentosum, an autosomal recessive disease characterized by a high incidence of skin cancers (Johnson *et al*., 1999b; Masutani *et al*., 1999). More recently, a novel DNA polymerase homologous to Pol β, named Pol lambda (λ), has been described and predicted to be involved in DNA repair synthesis during meiotic recombination (M.García-Díaz, O.Domínguez, L.A.López-Fernández, T.Laín de Lera, M.L.Saníger, J.F.Ruiz, M.Párraga, M.J.García, T.Kirchhoff, J.del Mazo, A.Bernad and L.Blanco, submitted).

A clear example of the existence of a particular enzymology for the generation of diversity is the enzyme terminal deoxynucleotidyltransferase (TdT), although its action appears to be restricted to specific genes such as those coding for antigen receptors (reviewed in Bentolila *et al*., 1995). TdT is a DNA-independent DNA polymerase, i.e. it has the ability to add nucleotides to DNA without any template information. This unusual ability is exploited at the broken ends of the V(D)J recombination intermediates of rearranging antigen receptor genes. The specificity of TdT action on these intermediates depends not only on its expression pattern, restricted to primary lymphoid organs, but also on specific interactions with the Ku protein, the DNA-binding component of DNA-dependent

protein kinase (DNA-PK), which functions in DNA repair, V(D)J recombination and isotype switching (Mahajan *et al*., 1999). Interestingly, TdT and Pol β are evolutionarily related, belonging to the family X of DNA polymerases (see Ito and Braithwaite, 1991).

We describe here the identification and preliminary biochemical characterization of a novel human DNA polymerase, named DNA polymerase mu (Pol μ), with a high similarity to TdT, and whose mRNA is highly expressed in secondary lymphoid tissues. The purified enzyme exhibited both terminal deoxynucleotidyltransferase activity and unprecedented error-proneness on primer–template structures. We propose that Pol μ could act as a DNA mutator polymerase responsible for the somatic hypermutation of immunoglobulin genes.

Results

Identification of a novel terminal transferase in human cells

The identification and cloning of the complete cDNA sequence of the novel human DNA polymerase Pol μ belonging to family X was carried out as described in Materials and methods. Pol μ is closely related to TdT, a member of the Pol X family whose template-independent polymerization capacity contributes to generation of diversity in antigen receptor genes (reviewed in Bentolila *et al*., 1995). Figure 1 shows a multiple alignment of human Pol μ and TdTs from different origins, demonstrating an overall amino acid identity of 41%. This value is significantly higher than that relating Pol μ and Pol β (23%) or Pol β and TdT (22%). A nuclear localization signal (NLS) of the most common type (SV40 large T antigen) is predicted at the sequence 'PKRRRAR' (residues 3–9) of Pol μ. A similar sequence in TdTs was proposed to act as an NLS (Bentolila *et al*., 1995). Interestingly, residues 22–120 of Pol μ are predicted (see Materials and methods) to form a BRCT domain (Bork *et al.*, 1997; Callebaut and Mornon, 1997). This domain, whose name derives from its initial identification at the C-terminal domain of the BRCA1 protein, is proposed to mediate protein–protein interactions in a variety of proteins involved in DNA repair and cell cycle checkpoint regulation upon DNA damage (Bork *et al*., 1997). As indicated in Figure 1, Pol μ residues 141–494 form a conserved Pol β core, whose three-dimensional structure in the presence of DNA and ddCTP has been solved at high resolution (Pelletier *et al*., 1994). Pol μ shares 139 of the 209 amino acid residues (66%) that are invariant among TdTs from very different origins, implying evolutionarily conserved structural and perhaps functional relationships. Pol μ also conserves 21 of the 23 amino acid residues that are conserved among all members of the heterogeneous Pol X family, including all those residues acting as metal, dNTP and DNA ligands, or triggering conformational changes upon ternary complex formation (see legend to Figure 1; for a review, see Oliveros *et al*., 1997). Therefore, as will be described later, characterization of the polymerization activity associated with Pol μ was necessary in order to determine whether this TdT homologue has terminal deoxynucleotidyltransferase activity.

Chromosomal mapping of human Pol μ

The human gene (*POLM*) coding for Pol μ was mapped initially to chromosome 7 by using a panel of human–rodent somatic cell hybrids (see Materials and methods). By radiation hybrid analysis (see Materials and methods), the SHGC marker that best linked with the *POLM* gene was SHGC-6115, with a lod score of 8.2. Based on the correspondence of this marker with the *GCK* gene, the *POLM* gene has been mapped within band 7p13. This region constitutes one of the four known fragile sites in lymphocytes, with a high incidence of molecular alterations such as deletions, inversions and translocations.

DNA polymerase activity associated with Pol μ

Human Pol μ was overproduced in *Escherichia coli* and purified to homogeneity as described in Materials and methods. The 55 kDa recombinant protein was obtained in soluble form in a high yield (see Figure 2A). Taking into account that family X DNA polymerases are low processive enzymes with no proofreading 3′–5′ exonuclease, assay conditions were selected to favour detection of a TdT-related enzyme such as Pol μ versus endogenous *E.coli* DNA polymerases. Thus, labelling of activated (gapped) heteropolymeric DNA was assayed in the presence of a low concentration of dATP as a single nucleotide, and activating Mn^{2+} ions. These conditions would favour incorporation of complementary and non-complementary nucleotide by terminal deoxynucleotidyltransferases (DNA independent), and by low-fidelity DNA-dependent DNA polymerases without proofreading activity. As shown in Figure 2B, under these conditions, DNA labelling with commercial TdT was ~10-fold more efficient using Mn^{2+} rather than Mg^{2+} activating ions; on the other hand, the opposite metal preference was obtained when labelling with the Klenow fragment of *E.coli* Pol I. As shown in Figure 2C, DNA labelling activity was detected in the 50% ammonium sulfate precipitate corresponding to the Pol μ-induced extracts, but it was very low in the corresponding uninduced fraction. Interestingly, the catalytic efficiency of the induced DNA polymerase was 20-fold higher in the presence of Mn^{2+} versus Mg^{2+} ions. This induced DNA polymerase activity was co-purified with the overproduced 55 kDa polypeptide throughout the purification procedure. As a further demonstration that the induced DNA polymerase activity was intrinsic to Pol μ, the heparin–Sepharose fraction (HS) was sedimented on a glycerol gradient. As shown in Figure 3, a DNA polymerase activity, preferentially activated by Mn^{2+} ions, co-sedimented perfectly at a molecular weight corresponding to the monomeric form of the Pol μ polypeptide. No 3′–5′ exo- or endonucleolytic activities were associated with the Pol μ peak (data not shown) and, therefore, the glycerol gradient fractions 9 and 10 (pooled) were used as the enzyme source for further activity assays.

Pol μ displays terminal deoxynucleotidyltransferase activity but requires a template–primer structure for optimal activity

In agreement with the structural similarity of Pol μ and TdT, a terminal deoxynucleotidyltransferase activity associated with Pol μ was demonstrated by using different oligonucleotides as single-stranded primer substrates, again in the presence of Mn^{2+} as the preferred cation.

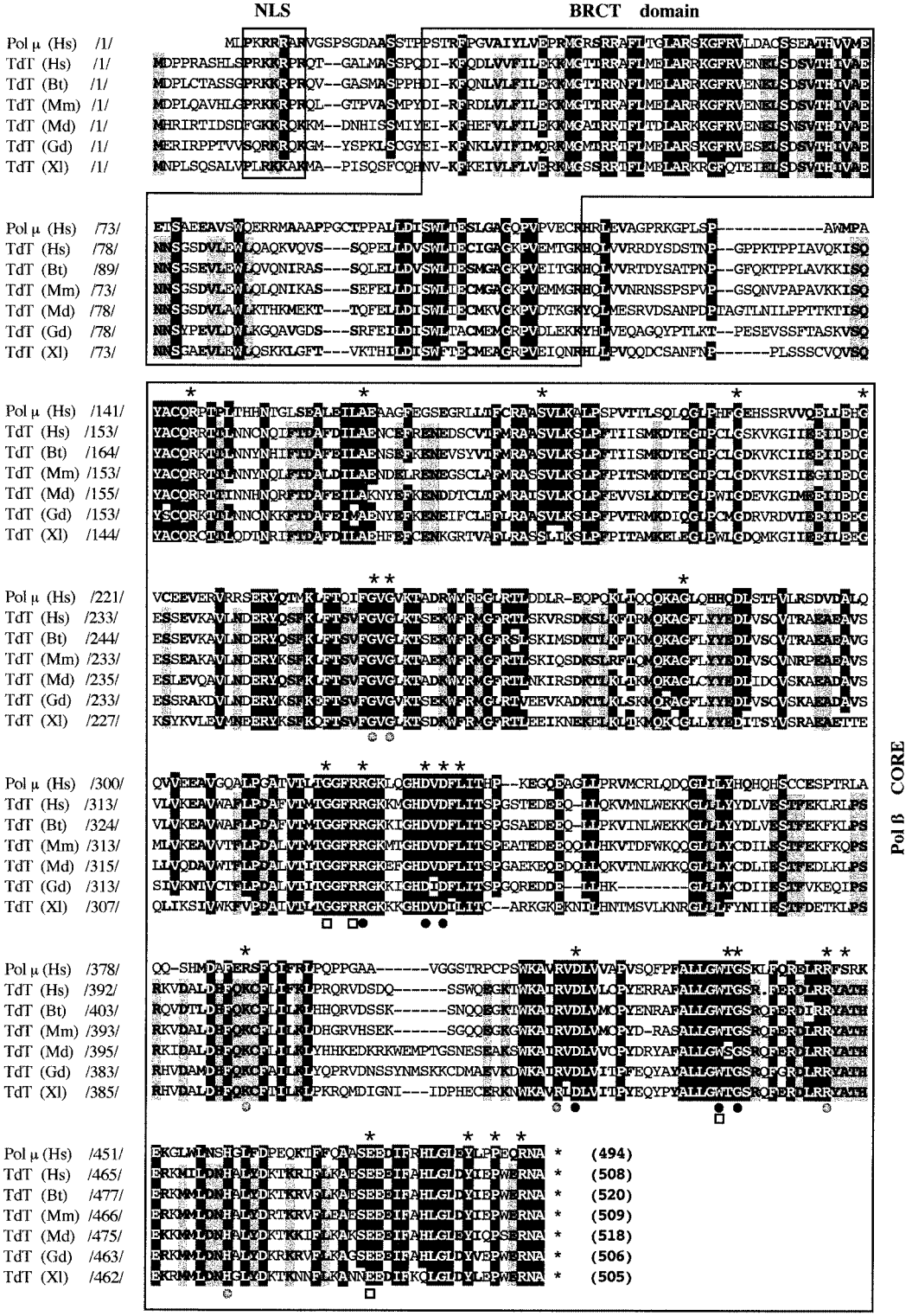

Fig. 1. Pol μ, a novel eukaryotic DNA polymerase homologous to TdT. Multiple alignment of human Pol μ (this study) with TdTs from human (Hs; sp P04053), bovine (Bt; sp P06526), murine (Mm; sp P09838), *Monodelphis domestica* (Md; sp Q02789), chicken (Gd; sp P36195) and *Xenopus laevis* (Xl; sp P42118). Numbers between slashes indicate the amino acid position relative to the N-terminus of each DNA polymerase. A putative nuclear localization signal (NLS) at residues 3–9 of human Pol μ is boxed. Amino acid residues 22–118 of Pol μ (boxed) are predicted to form a BRCT domain (Bork *et al*., 1997). Amino acid residues 141–494 of Pol μ (boxed) form a conserved Pol β core (see text for details). Invariant residues between Pol μ and TdTs are indicated with white letters (on a black background). Identical residues among TdTs are in bold and boxed (grey). Other relevant similarities between Pol μ and TdTs are in bold. Conservative substitutions were considered as follows: K, H and R; D, E, Q and N; W, F, Y, I, L, V, M and A; G, S, T, C and P. The 23 residues that are invariant among DNA polymerase X members (Oliveros *et al*., 1997) are indicated with an asterisk. Dots at the bottom of the alignment indicate putative homologues to Pol β residues (Pelletier *et al*., 1994) shown to act either as DNA ligands (Gly64, Gly66, Gly105, Gly109, Lys234, Arg254, Arg283 and Tyr296; grey), or as dNTP and metal ligands (Phe272, Gly274, Arg183; Asp190, Asp192 and Asp256; black). Squares at the bottom of the alignment indicate putative homologues to Pol β residues involved in interactions between the 'palm' and 'thumb' subdomains (Gly179/Phe272; Arg182/Glu316). The total length, in number of amino acid residues, is indicated in parentheses.

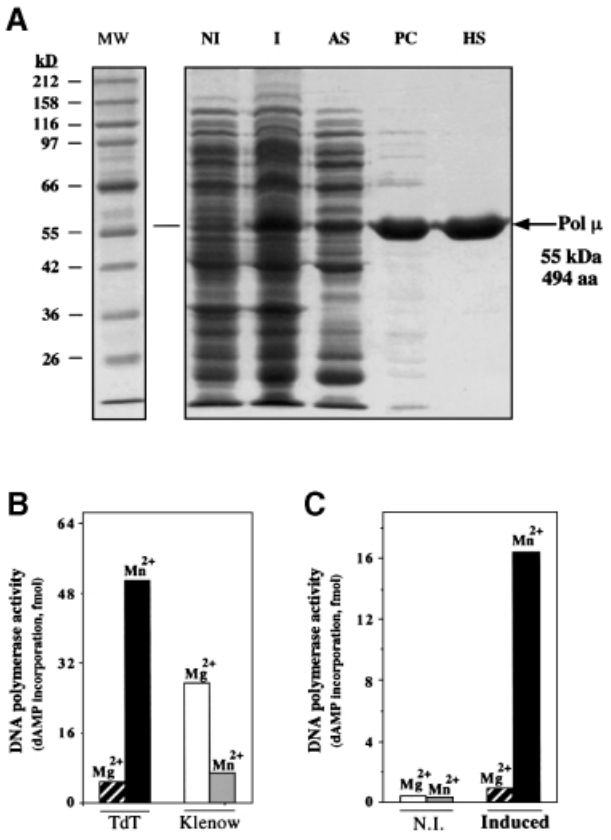

Fig. 2. Expression of human Pol μ in *Escherichia coli*. (**A**) Coomassie Blue staining after SDS–PAGE separation of control non-induced (NI) and IPTG-induced (I) extracts of *E.coli* BL21(DE3) cells transformed with the recombinant plasmid pRSET-hPolμ, and further purification steps of the latter extracts. The mobility of the induced protein Pol μ was compatible with its deduced molecular mass (55 kDa/494 amino acids). After PEI precipitation of the DNA, Pol μ was precipitated with 50% ammonium sulfate (AS), and purified further by phosphocellulose (PC) and heparin–Sepharose (HS) chromatography, as described in Materials and methods. The electrophoretic migration of a collection of molecular mass markers (MW) is shown at the left. (**B**) Relative activation by Mg^{2+} versus Mn^{2+} of TdT and Klenow enzymes during DNA polymerization ([α-^{32}P]dATP labelling) on activated DNA. TdT (5 U) and Klenow (1 U) were assayed for 30 min at 37°C, in the presence of either 10 mM $MgCl_2$ or 1 mM $MnCl_2$ as a source of activating metal ions. DNA polymerase activity, expressed as dAMP incorporation, was quantitated as described in Materials and methods. (**C**) DNA polymerization activity associated with Pol μ expression. The 50% AS fraction corresponding to either non-induced (N.I.) or induced extracts was assayed and quantitated as described in (B).

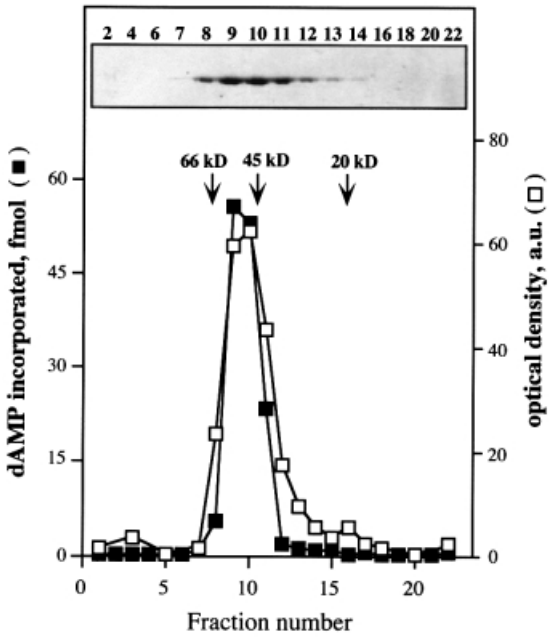

Fig. 3. Co-sedimentation of a DNA polymerase activity with the Pol μ polypeptide. The heparin–Sepharose fraction (HS) shown in Figure 2A was sedimented on a glycerol gradient (15–30%) and fractionated as described in Materials and methods. The inset shows an SDS–PAGE analysis followed by Coomassie Blue staining of some selected fractions. Fractions are numbered from the bottom (1) to the top (22). Arrows indicate the sedimentation position of several molecular mass markers centrifuged under identical conditions. Quantitation of the Pol μ band corresponding to each fraction is expressed in arbitrary units of optical density (a.u.; right ordinates). DNA polymerase activity ([α-^{32}P]dATP labelling of activated DNA) of each fraction, assayed for 15 min at 37°C in the presence of 1 mM $MnCl_2$ (see Materials and methods), is expressed as dAMP incorporation (left ordinates).

As shown in Figure 4A, Pol μ was able to catalyse polymerization of any of the four dNTPs to a single-stranded DNA primer in the absence of a template. The catalytic efficiency of the terminal deoxynucleotidyltransferase activity of Pol μ varied as a function of the nucleotide used, dTTP and dCTP (both pyrimidines) being inserted the most efficiently, and dATP the least efficiently. A different nucleotide preference was observed when using TdT, dGTP and dCTP being the preferred nucleotide substrates under these conditions. The terminal deoxynucleotidyltransferase associated with Pol μ was also active, although less efficient, on double-stranded DNA substrates where the primer terminus was paired with a 5′-terminal complementary nucleotide (blunt-ended), a behaviour already described for TdT (results not shown).

Interestingly, the level of Pol μ-catalysed dTMP incorporation obtained on single-stranded substrates such as oligo(dT) or poly(dA), assayed independently, increased up to 370-fold when these were pre-hybridized to form a template–primer structure (Figure 4B). On the contrary, TdT catalysed a similar incorporation on both single-stranded homopolymers and a poly(dA)/oligo(dT) substrate, in agreement with its template independence (data not shown). Therefore, and in spite of its intrinsic terminal deoxynucleotidyltransferase activity, Pol μ may be defined as a DNA-dependent DNA polymerase, since it requires a template–primer for optimal activity.

Pol μ is an error-prone DNA-dependent DNA polymerase

When the polymerization assay on activated DNA (used to monitor Pol μ activity during purification) was carried out in the presence of all four deoxynucleotides, incorporation of the labelled dATP substrate by Pol μ was strongly inhibited (Figure 5A). In fact, under the standard conditions used to assay most DNA polymerases (>100-fold unlabelled versus labelled nucleotide precursors), Pol μ activity would not be detectable. A similar inhibition was obtained with TdT whereas, in the case of the Klenow enzyme, an increase (11-fold) in dAMP incorporation was obtained by addition of all four nucleotide substrates, as expected. Moreover, Pol μ-catalysed dAMP incorporation on poly(dT)/oligo(dA) was also inhibited strongly by rela-

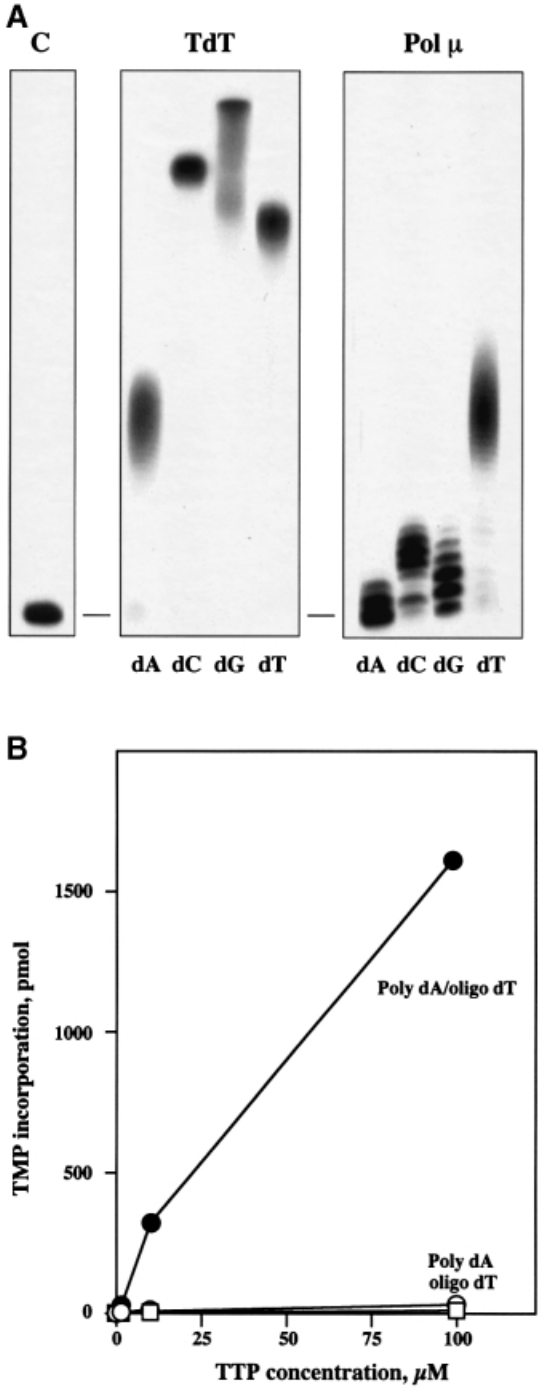

Fig. 4. Pol μ has terminal transferase activity, but requires a template–primer structure for optimal efficiency. (**A**) Terminal transferase activity associated with human Pol μ. The assay was carried out as described in Materials and methods, using 3.2 nM 5′-labelled single-stranded 19mer (P19) as substrate, 1 mM $MnCl_2$ as a source of activating metal ions, 80 μM each individual deoxynucleotide, and either TdT (2.5 U/41 ng) or Pol μ (20 ng). A control reaction in the absence of enzyme (C) was also carried out. After incubation for 30 min at 30°C, extension of the 5′-labelled oligonucleotide was analysed by 8 M urea–20% PAGE and autoradiography. (**B**) Template-dependent polymerization catalysed by Pol μ. Polymerization efficiency was assayed comparatively on either poly(dA) (○), oligo(dT) (□) or a poly(dA)/oligo(dT) hybrid (●) to provide a homopolymeric template (dA)n. The assay was carried out in the presence of 1 mM $MnCl_2$, 13 nM [α-^{32}P]dTTP, Pol μ (20 ng) and 0.5 μM each DNA substrate. After incubation for the indicated times at 37°C, dTMP incorporation was quantitated as described in Materials and methods.

tively low concentrations of any of the other three (non-complementary) nucleotides (Figure 5B). An identical behaviour was obtained by using TdT in a parallel assay (results not shown). On the contrary, non-complementary nucleotides did not inhibit polymerization by the Klenow enzyme (see Figure 5B). These results suggest that dAMP incorporation by Pol μ is being competed by the other nucleotides, as would be expected either for a template-independent terminal transferase such as TdT, or for a DNA-dependent DNA polymerase with a poor template-directed nucleotide discrimination.

The ability of Pol μ to select among the four deoxynucleotides (base discrimination) to catalyse faithful template-directed DNA synthesis was evaluated initially on the four template–primer structures depicted in Figure 6, obtained as described in Materials and methods. The four dNTPs, at varying concentrations, were assayed individually as a substrate for each of the four template–primer structures, thus representing the 16 possible template–substrate nucleotide pairs (four matched + 12 mismatched). The same primer molecule (without any template) was assayed in parallel to estimate the residual terminal transferase activity of Pol μ with each of the four dNTPs. As shown in Figure 6, under the conditions used in this experiment, only dTMP was incorporated, with either Mg^{2+} or Mn^{2+}, into the single-stranded DNA primer. On the contrary, on the four template–primer structures, preferential insertion of the nucleotide complementary to the first template base was observed in the presence of Mg^{2+} ions, indicating that the catalytic efficiency (K_{cat}/K_m) was improved greatly by template selection of the incoming nucleotide (note that the complementary nucleotide is provided at a 10-fold lower concentration than that used in the control without template). However, template instructions appear not to be very rigorous, since the enzyme is able to add non-complementary nucleotides at 100 μM (dT and dA in Figure 6), and non-complementary dCTP at higher concentrations (data not shown). The probability of G:A misincorporation was estimated to be only 10- to 50-fold lower than that of a correct G:C pair (data not shown). Considering the efficiency of dTMP incorporation on single-stranded DNA, it cannot be ruled out that the observed insertion of dTMP using non-complementary templates might be due to residual amounts of non-hybridized primer.

In the presence of Mn^{2+} as metal activator, the pattern and efficiency of nucleotide incorporation changed drastically (see Figure 6). In all cases, dNTP incorporation was driven by the presence of a template DNA (note that the nucleotide concentration was 1000-fold lower than that used for Mg^{2+} activation), but with a poor or null base selectivity. As an example, when dC is the first template base, the four dNTPs appear to have similar probabilities of being inserted. Exceptionally, dGTP incorporation occurred mainly in front of its complementary nucleotide. Moreover, inserted errors are elongated efficiently, favoured not only by complementarity but also as reiterative misinsertions, particularly when using dTTP and dATP substrates. In the same assay, a Pol β-like enzyme of only 20 kDa (ASFV Pol X) was shown to extend the four template–primer structures by adding only the correct (complementary) deoxynucleotide, but not by adding an excess (400 μM) of each of the three incorrect (non-

complementary) deoxynucleotides (Oliveros *et al.*, 1997). Similar results were obtained when Mn^{2+} was used instead of Mg^{2+} as metal activator (results not shown).

All these results demonstrate that Pol μ is an error-prone DNA-dependent DNA polymerase. Interestingly, in the presence of its preferred activator (Mn^{2+}), Pol μ behaves as a strong mutator, lacking base discrimination during nucleotide insertion on a DNA template–primer structure. Exceptionally, Pol μ preferentially inserts a dG in front of its complementary dC template base even in the presence of Mn^{2+} ions.

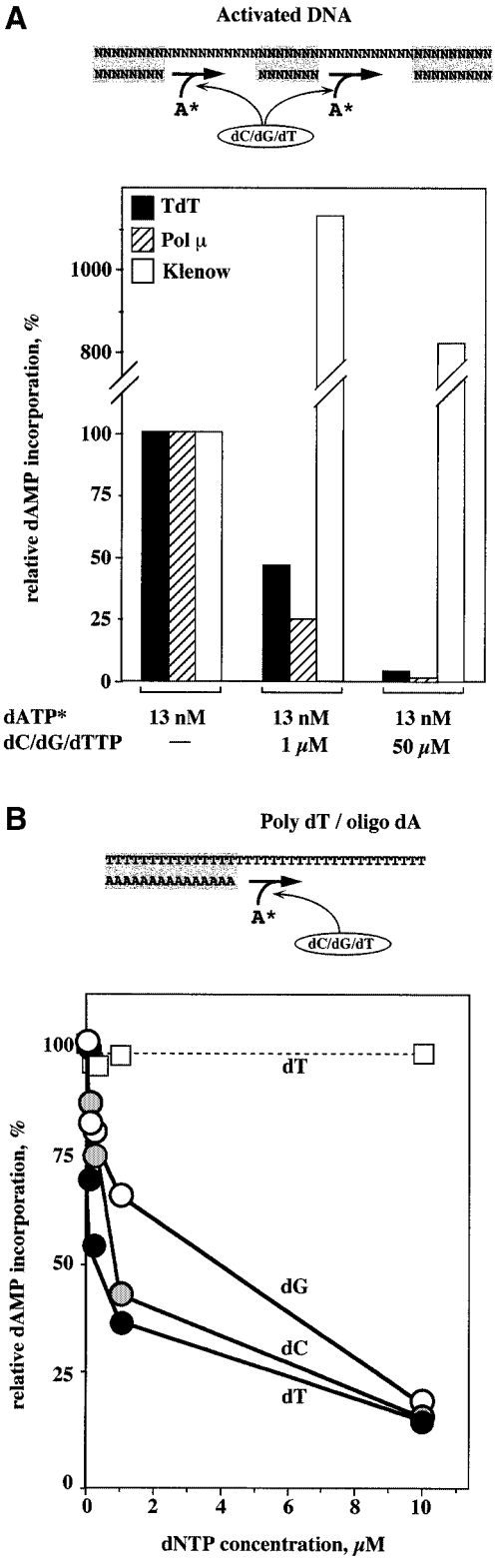

Fig. 5. Inhibition of DNA-directed synthesis by non-complementary dNTPs. (**A**) Inhibition of [α-^{32}P]dATP labelling of activated (gapped) DNA by addition of different concentrations of a mixture of dC, dG and dTTP, in the presence of 1 mM $MnCl_2$ (a scheme is depicted). Under the standard conditions described in Materials and methods, only dATP (13 nM) is used as substrate for this assay. After incubation for 15 min at 37°C in the presence of either TdT (2.5 U/41 ng), Klenow (1 U) or Pol μ (20 ng), and the concentration indicated of dNTPs, dAMP incorporation on activated DNA was expressed as a percentage of that obtained under standard assay conditions: 100% represents either 73 (TdT), 13 (Klenow) or 8 (Pol μ) fmol of incorporated dAMP. (**B**) A similar analysis was carried out, but using a poly(dT)/oligo(dA) hybrid to provide a homopolymeric template (dT)n. The assay was carried out in the presence of 1 mM $MnCl_2$, 13 nM [α-^{32}P]dATP as the correct nucleotide, either 20 ng of Pol μ (circles) or 1 U of Klenow (squares), and the concentration indicated (on the abscissa) of individual non-complementary dNTPs. After 5 min at 37°C, dAMP incorporation on poly(dT)/oligo(dA) was expressed as a percentage of that obtained when non-complementary nucleotides were added: 100% represents either 23 (Pol μ) or 127 (Klenow) fmol of incorporated dAMP.

Pol μ mRNA is expressed predominantly in peripheral lymphoid tissues

Quantitative analyses of Pol μ transcription levels in different human tissues were carried out by Northern blotting using commercial membranes containing normalized amounts of poly(A)$^+$ RNA from different human tissues (see Materials and methods). As shown in Figure 7, a major transcript migrating at ~2.6 kb, in agreement with the size of the cDNA isolated (2589 nucleotides), was accumulated at the highest level in lymph nodes, followed by spleen, thymus, pancreas and peripheral blood lymphocytes. Lower levels of this transcript were present in the other tissues examined, being undetectable only in lung.

By searching the expressed sequence tag (EST) database using Pol μ cDNA, we identified a collection of 40 ESTs corresponding to human Pol μ; 37% of these ESTs derive from different tumours. It is worth noting that 36% of the non-tumoural ESTs of Pol μ derive from human tonsillar cells enriched for germinal centre B cells (CD20$^+$, IgD$^-$) by flow sorting (library NCI-CGAP-GCB1). The significance of this finding is higher considering the fact that only 7% of the available ESTs corresponding to Pol β, a housekeeping DNA repair polymerase, derive from germinal centre B cells. All these data suggested that Pol μ mRNA could be expressed preferentially in human B cells, particularly in populations associated with the germinal centre structures present in secondary lymphoid organs. To confirm this suggestion, we used *in situ* hybridization to analyse the localization, at the cellular level, of the Pol μ mRNA present in different human tissues. Using a specific antisense probe corresponding to the first 1200 nucleotides of Pol μ cDNA (see Materials and methods), expression of Pol μ mRNA was observed in tissue sections corresponding to human secondary lymphoid organs. Thus, as shown in Figure 8, and in agreement with the data obtained by Northen blotting, the stronger signal was found in peripheral lymph node

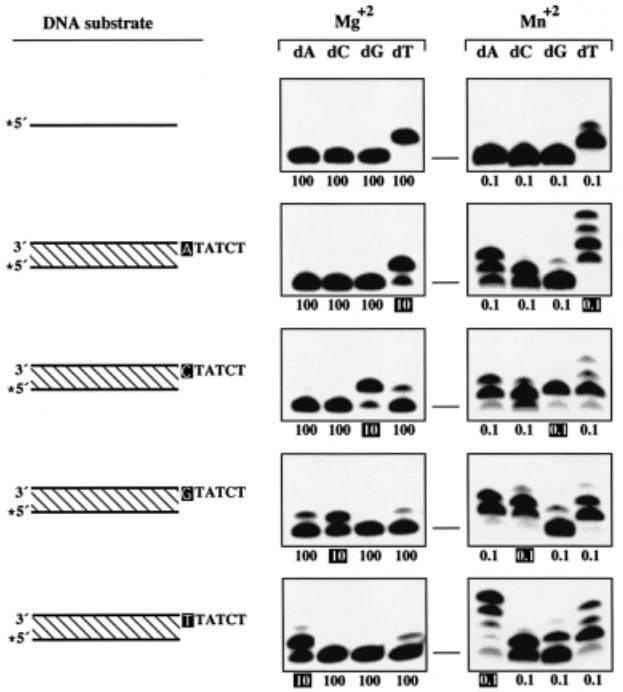

Fig. 6. Pol μ-catalysed misinsertion at the four template bases. The four template–primer structures used, which differ only in the first template base (outlined), are indicated on the left. The single-stranded oligonucleotide corresponding to the primer strand was assayed in parallel as a control of DNA-independent nucleotide insertion. Mg^{2+}-activated nucleotide insertion on each 5′-labelled DNA substrate (3.2 nM) was analysed in the presence of either the complementary nucleotide (10 μM) or each of the three incorrect dNTPs (100 μM), as described in Materials and methods. Mn^{2+}-activated nucleotide insertion was assayed with each of the four dNTPs (0.1 μM). After incubation for 15 min at 30°C in the presence of 20 ng of human Pol μ, extension of the 5′-labelled (*) strand was analysed by electrophoresis in an 8 M urea–20% polyacrylamide gel and autoradiography.

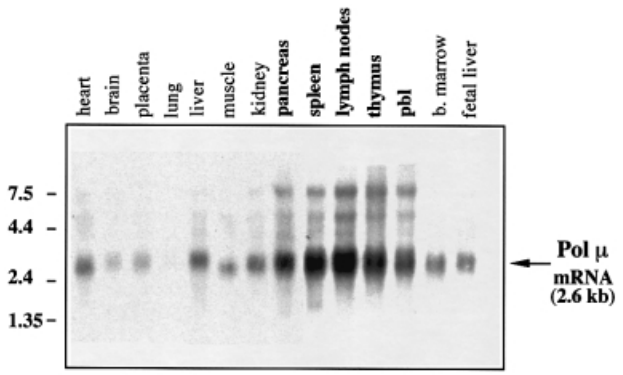

Fig. 7. Pol μ mRNA is expressed preferentially in secondary lymphoid organs. Northern blotting analysis of TdT-2 mRNA was carried out as indicated in Materials and methods, using commercial blots (MTN and MTN-II blots, Clontech) containing poly(A)$^+$ RNA from the human tissues indicated. The membrane was hybridized with a specific ^{32}P-labelled DNA probe containing 1141 nucleotides of the Pol μ cDNA 3′-terminal sequence. The hybridized probe, revealing a major transcript (2.6 kb), was detected by autoradiography.

sections; a strong signal was also found in sections from spleen. Hybridization specificity was assessed by using a sense riboprobe under the same experimental conditions and in a close parallel tissue section, not producing a comparable signal (see Figure 8). In similar experiments, other human organs such as muscle, lung or even bone marrow (the latter shown in Figure 8), a myelo-lymphoid tissue, were negative or faintly positive in comparison with the corresponding negative control. As shown in Figure 8, the level of expression of the Pol μ mRNA in lymph nodes seems to be high, in comparison with other markers for centroblastic populations such as A-myb (data not shown). The *in situ* hybridization pattern obtained is compatible with a preferential expression in the follicular lymphoid region, with a variable expression in different areas and not restricted to a particular cell subpopulation. The expression level of Pol μ mRNA in spleen is lower in comparison with that observed in lymph nodes, and more restricted to particular structures, such as that shown in Figure 8, which resemble the typical organization of a germinal centre in a secondary follicular area. Also in this case, expression of Pol μ mRNA does not seem to be associated preferentially with a discrete cell subpopulation. In summary, it seems that Pol μ mRNA is expressed preferentially in the follicular areas of human secondary lymphoid organs. Therefore, although immunostaining of Pol μ in similar tissue sections is still lacking, it is tempting to speculate that Pol μ expression could be associated with the generation, in these organs, of germinal centres, specialized structures that are critical for the maturation of the immune humoral response.

Discussion

The amino acid sequence derived from the Pol μ cDNA (a total of 494 amino acids) showed 41% identity with TdTs from different origins, suggesting that Pol μ could be involved in specific processes such as V(D)J recombination of Ig genes, in which TdT contributes to variability by adding non-template nucleotides. Interestingly, Pol μ contained 21 of the 23 residues that are invariant in all DNA polymerases from family X, including all those involved in DNA and nucleotide binding, catalysis of polymerization and conformational changes involved in the polymerization cycle, as defined for Pol β, the paradigmal enzyme for DNA polymerase family X. In order to delineate the function of Pol μ, we carried out a preliminary biochemical characterization of the human Pol μ protein overproduced in *E.coli*, together with expression analysis in different tissues at the RNA level.

Pol μ, a mutator DNA polymerase

The results presented here demonstrate that Pol μ shares enzymatic properties with different members of the Pol X family. Like TdT, its closest homologue, Pol μ has an intrinsic terminal transferase activity that preferentially inserts pyrimidine nucleotides. Thus, its relative nucleotide usage, different from that observed for TdT, is: dT>>dC>dG>dA. As for Pol β and other DNA-dependent DNA polymerases, but unlike TdT, the catalytic efficiency of nucleotide incorporation by Pol μ was greatly enhanced by the presence of a template strand. Both the terminal transferase and DNA-dependent DNA polymerization activities of Pol μ are strongly activated *in vitro* by manganese ions. Interestingly, in the presence of its preferred activator (Mn^{2+}), Pol μ behaves as a strong mutator, lacking any base discrimination during nucleotide insertion.

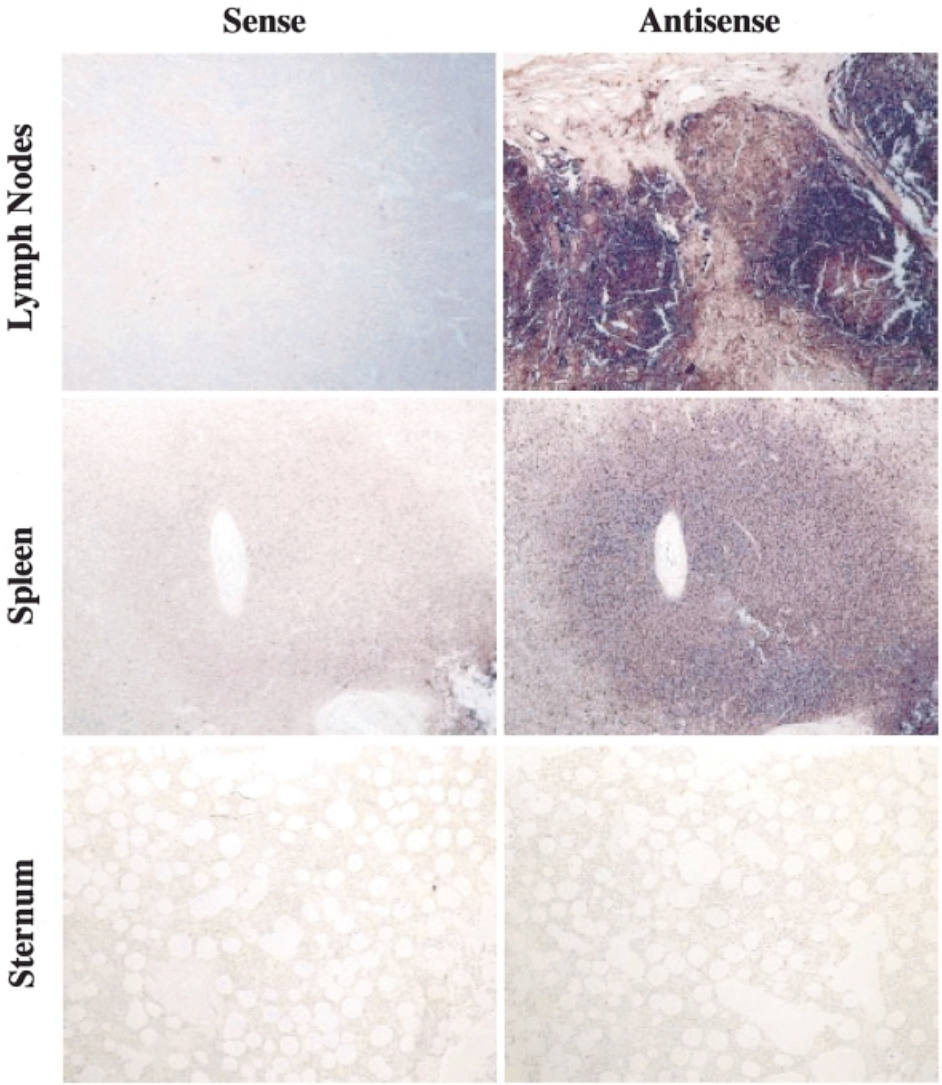

Fig. 8. *In situ* hybridization of Pol μ mRNA in different human tissues. DIG-labelled sense and antisense riboprobes, corresponding to the first 1200 nucleotides of Pol μ cDNA, were obtained and hybridized to human tissue sections (Human Tissue Set I and Human Hematal and Immune Tissue Set, Novagen) under the conditions described in Materials and methods. After hybridization, detection of the RNA probes in tissue sections was carried out by incubation with anti-DIG–alkaline phosphatase antibody.The dark blue staining, observed in lymph nodes and spleen with the antisense riboprobe, outlined regions largely expressing Pol μ mRNA. No comparable signal was obtained by using a sense riboprobe under the same experimental conditions and in a close parallel tissue section.

Structural studies demonstrated that two metal ions, coordinated by a triad of carboxylate residues, are required at the active site of polymerases (for a review, see Joyce and Steitz, 1994). Although Mg^{2+}-activated catalysis has been the form studied most extensively, other metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+} can also serve as DNA polymerase activators *in vitro*, although it is unknown at present which metal or metals are used *in vivo*. The mutagenicity of Mn^{2+} and other transition metal ions such as Co^{2+}, by reducing the nucleotide selectivity of many DNA polymerases, has been recognized for some time (Weymouth and Loeb, 1978; Goodman *et al*., 1983; El-Deiry *et al*., 1984). In addition to metal–DNA interactions (Murray and Flessel, 1976) that could affect DNA polymerization fidelity (Goodman *et al*., 1983; Beckman *et al*., 1985), it has been shown that metals other than Mg^{2+} affect orientation of one of the catalytic aspartates in Pol β crystals (Pelletier *et al*., 1996). There is also crystallographic evidence in Pol β that initial binding of a nucleotide from solution is non-base dependent, occurring primarily via the triphosphate and sugar moieties (Pelletier *et al*., 1996). These authors argue that in the presence of Mg^{2+}, the initial non-selective nucleotide-binding step could be weak and transient, with a diffusion rate too fast to affect overall catalysis; however, an increased stability mediated by Mn^{2+} ions would result in the three incorrect nucleotides being competitive inhibitors of the correct nucleotide. Such an Mn^{2+}-stimulated non-selective step could be an important factor contributing to the mutator capacity of Pol μ, as could be illustrated by the experiment shown in Figure 5.

It has also been suggested that another way in which Mn^{2+} may exert its mutagenic effect on polymerases is by promoting greater reactivity than Mg^{2+} at the catalytic site, thereby allowing the nucleotidyl transfer reaction to take place with little or no need for instructions from a template (Pelletier *et al*., 1996). In this sense, it has been reported that most non-proofreading DNA polymerases,

including Pol β, are able to catalyse a Mn^{2+}-driven single (+1) addition to blunt-ended DNA substrates (Clark, 1988). Such a reaction occurred even in Pol β crystals having Mn^{2+} at its active site, providing a structural basis for metal ion mutagenicity and nucleotide selectivity extrapolatable to most DNA polymerases (Pelletier *et al*., 1996). Moreover, some specific reactions, such as the TP-primed initiation of ϕ29 DNA replication carried out by ϕ29 DNA polymerase, are greatly enhanced (100-fold) when Mn^{2+} ions are used instead of Mg^{2+} ions (Esteban *et al*., 1992).

Although a detailed kinetic analysis of Mn^{2+} activation is still lacking, the results presented here support the idea that Pol μ takes the maximal advantage of Mn^{2+} ions to activate the template-dependent addition of nucleotides with a poor or null base selectivity. Further studies of Pol μ mutator capacity will be required in order to establish the preferred and accessible DNA structures among DNA ends versus nicked, gapped, flapped or mismatched DNA, and to analyse the specificity of the mutations introduced in each case. It will be interesting to test whether the extent of the reaction and spectra of mutations will be influenced by the presence of DNA-PK, as has been reported to be the case for nucleotidyl transfer by TdT (Mickelsen *et al*., 1999). Special attention should be paid to those sequences known as hot spots or target sequences for somatic hypermutation (see below). Finally, the availability of large amounts of soluble Pol μ, overproduced in *E.coli*, will allow us to initiate structural studies aimed at determining the molecular basis of Pol μ mutagenicity.

Pol μ, a candidate enzyme for somatic hypermutation of Ig genes

During B-cell development, V(D)J recombination in pro-/pre-B cells of the bone marrow creates the primary repertoire of antibody specificities. Following antigen encounter, the rearranged V genes of those cells that have been triggered by the antigen are subjected to a second mechanism for affinity maturation and further specificity diversification, known since its first description in 1970 as somatic hypermutation (Weigert *et al.*, 1970). There is strong evidence in mouse, man and sheep that the germinal centres formed at secondary lymphoid organs are the site wherein antigen-stimulated B cells acquire somatic mutations (Berek *et al*., 1991; Jacob *et al*., 1991). Hypermutation introduces an estimated 10^{-3}–10^{-4} point mutations (per base pair per generation) specifically into the variable (V) regions of the gene segments coding for Igs, being ~10^6-fold higher than the spontaneous mutation rate operating in the rest of the genome (reviewed by Neuberger and Milstein, 1995). It has been reported that Ig hypermutation exhibits a distinctive pattern of nucleotide misincorporations favouring transition mutations (Golding *et al*., 1987), and targeting G:C base pairs preferentially (Betz *et al*., 1993; Bachl and Wabl, 1996). Several di- or trinucleotides have been defined as hot spots (Smith *et al*., 1996), the most important being the G:C base pair embedded in the motifs CAGCT and AAGTT (Betz *et al*., 1993, 1994) and short palindromes or hairpin loops (Levy *et al*., 1988; González-Fernández *et al*., 1994). Very recently, analysis of the sequences around individual nucleotide substitutions in IgV_H genes suggested the existence of different GC and AT mutators (Spencer *et al*., 1999).

The original model proposed that somatic hypermutation is triggered by the generation of single- or double-stranded breaks in the V region followed by an error-prone repair mechanism (Brenner and Milstein, 1966). More recently, the study of lymphoid cell lines that undergo a constitutive (Sale and Neuberger, 1998) or inducible (Denépoux *et al*., 1997; Zan *et al*., 1999) hypermutation allowed the demonstration that such DNA strand breaks are scattered specifically within the V mutation domain (Sale and Neuberger, 1998). Although dissection of the mechanism involved in somatic hypermutation has produced important clues about *cis*-acting factors, the enzymatic activities involved in the process are still a matter of speculation (reviewed by Storb *et al*., 1998). A reverse transcriptase activity was proposed to be involved in the process because of its low level of fidelity (Steele and Pollard, 1987), although no further experimental support has been reported. Recently, it has been proposed that somatic hypermutation takes place by an error-prone and short patch DNA synthesis process occurring outside of global semiconservative DNA replication (Bertocci *et al*., 1998). This proposal also agrees with recent reports that post-replicative mismatch repair is neither involved primarily in the hypermutation process (Frey *et al*., 1998; Jacobs *et al*., 1998; Phung *et al*., 1998; Winter *et al*., 1998) nor is it merely a co-option mechanism (Cascalho *et al*., 1998). Based on all this evidence, a candidate mutagenic DNA polymerase, functionally analogous to Pol β and 'copying' very short segments of DNA, has been invoked (Bertocci *et al*., 1998).

TdT was the first candidate proposed to be a somatic mutagen in lymphocytes (Baltimore, 1974). Twenty-four years later, it has been demonstrated that the DNA strand breaks specifically occurring at the V segments of Ig genes are accessible to TdT when this enzyme is transfected into the Ramos cell line (Sale and Neuberger, 1998). As shown here, a novel TdT-like DNA polymerase, Pol μ, is expressed preferentially in secondary lymphoid tissues, and a large proportion of the ESTs corresponding to this polymerase derive from germinal centre B cells. This circumstantial evidence, together with the catalytic properties of Pol μ as a strong mutator DNA polymerase, makes this enzyme a suitable candidate to participate in somatic hypermutation of Ig genes.

Concluding remarks

We have recently obtained the full-length cDNA corresponding to the murine homologue of Pol μ. In agreement with the data shown here, the mRNA for murine Pol μ was abundant in secondary lymphoid tissues, although basal levels were detected in most tissues examined (our unpublished results). Moreover, *in situ* analysis of Pol μ mRNA expression in spleen from immunized mice showed a clear association with developing germinal centres (our unpublished results). The hypothesis that Pol μ is a mutator of immunoglobulin genes is being tested by transfection of a Ramos cell line with a catalytically inactive Pol μ mutant, expected to produce a dominant-negative inhibition of ongoing somatic hypermutation. Characterization of the murine locus for Pol μ (our unpublished data) has

allowed us to initiate the generation of a knock-out model for studying Pol μ deficiency.

Materials and methods

Nucleotides and proteins

Unlabelled nucleotides were purchased from Pharmacia P-L Biochemicals. [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol) and [α-^{32}P]dTTP (3000 Ci/mmol) were obtained from Amersham International Plc. Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase and TdT were from Boehringer Mannheim. Klenow fragment was from New England Biolabs. *Taq* DNA polymerase was from Perkin Elmer, and *Pfu* DNA polymerase was from Stratagene. MMLV reverse transcriptase was obtained from Gibco-BRL. ASFV Pol X was kindly provided by M.Oliveros (Madrid).

Oligonucleotides, DNA templates and substrates

Oligonucleotides used for cDNA cloning were: S2G (5′-ACAGGGGGGTTCCGGAGGGG); ASH2 (5′-AAAAATGTCTTCTGCTCCGG); h2asR (5′-CAGGCGGCACATCACTCT); h2sP (5′-CTCTGAGCCCAGCATGGA); h2sR (5′-GAAGTTGCAGGGCCATGAC); h2asZ (5′-CCTCGCCTAACAAAGTGGC); h2NdeATGs (5′-GCTGTCGTCCATATGCTCCCCAA); h2asQ3 (5′-GTCATGGCCCTGCAACTT); h2sQ2 (5′-GAGGTACCAGACCATGAAGCTC); and h2ER1TGAas (5′-CGGAATTCAGGCGTTTCTCTGCTC). Oligonucleotides used for chromosomal mapping were: h2MAPs (5′-GCCACTGAATGTCTCCAAGC) and h2MAPas (5′-TGCAGTGCAGGTATGCATGG). Oligonucleotides used as primer strands for functional assays were: P15 (5′-GATCACAGTGAGTAC); P19 (5′-GATCACAGTGAGTACAATA); oligo(dA)$_{15}$; and oligo(dT)$_{10}$. Oligonucleotide T15c+6 (5′-TCTATTGTACTCACTGTGATC-3′), which has a 5′-terminal extension of six nucleotides in addition to the sequence complementary to P15, oligonucleotides T15c+6(A) (5′-TCTATAGTACTCACTGTGATC), T15c+6(G)(5′-TCTATGGTACTCACTGTGATC) and T15c+6(C) (5′- TCTATCGTACTCACTGTGATC), differing in the first template base (indicated in their respective names) with respect to the sequence of T15c+6, poly(dA) and poly(dT) were used as template strands. Oligonucleotide P19c (5′-TATTGTACTCACTGTGATC), complementary to P19, was used to make a blunt-ended DNA molecule. Oligonucleotides were obtained from Genset and Gibco-BRL. Oligonucleotides SP1, oligo(dA)$_{15}$ and oligo(dT)$_{10}$ were 5′-labelled with [α-^{32}P]ATP and T4 polynucleotide kinase and purified by electrophoresis on 8 M urea–20% polyacrylamide gels. To analyse the DNA-dependent polymerization activity of the protein on different primer–template structures, the labelled oligonucleotides (primer) were hybridized to partially complementary 5′-protruding oligonucleotides (template) in the presence of 0.2 M NaCl and 60 mM Tris–HCl pH 7.5. Human placenta poly(A)$^+$ RNA, obtained from Clontech, and total RNA from Ramos cells, obtained according to the Tri-Reagent (Sigma) kit instructions, were used as templates for reverse transcription with MMLV reverse transcription.

Cell lines

Ramos cells were maintained in RPMI 1640 (Gibco-BRL), supplemented with 10% fetal calf serum (Gibco-BRL), 2 mM L-glutamine (Merck), streptomycin (0.1 mg/ml; Sigma) and penicillin (100 U/ml; Sigma), at 0.2–1 × 10^6 cells/ml. These cells, derived from a Burkitt's lymphoma, constitutively diversify their rearranged Ig V gene during *in vitro* culture, without the requirement for stimulation by activated T cells, exogenously added cytokines or even maintenance of the B-cell antigen receptor (Sale and Neuberger, 1998).

Identification and gene cloning of human Pol μ

An EST clone (DDBJ/EMBL/GenBank accession No. AA298793) containing the partial sequence of a putative new DNA polymerase was identified by a GAPPED-BLAST (Altschul *et al*., 1997) search of the NCBI EST database using as probes different conserved amino acid segments belonging to the catalytic core of family X DNA polymerases, derived from the alignment reported by Oliveros *et al.* (1997). This new putative polymerase, named Pol μ, fulfils the consensus pattern: **G**-[**SG**]-[L**F**Y]-**x**-**R**-[**G**E]-**x(3)**-[S**G**CL]-**x**-**D**-[LI**V**M]-**D**-[**LI**VM**FY**] (**3**)-**x(2)**-[SA**P**], corresponding to the DNA polymerase X signature (PROSITE: PDOC00452). The human Pol μ cDNA sequence was obtained through a series of overlapping cloning steps. First, the EST clone AA298793, identified by BLAST analyses, was obtained from the IMAGE Consortium (Lennon *et al*., 1996; http://www-bio.llnl.gov/bbrp/image/image.html), sequenced and shown to contain 1141 nucleotides corresponding to the 3′ end of the putative Pol μ. A segment of 5′ upstream sequence was obtained by PCR on placenta cDNA from a specific antisense primer derived from the EST clone (ASH2) and a degenerate sense primer derived from the most conserved coding portions of the TdT gene family (S2G). PCR was performed with a standard profile of 95°C/15 s, 62°C/15 s and 72°C with an extension time of 1 min per kb to be amplified and a number of cycles defined by the template concentration. Reaction primers were used at 1 μM, and either *Taq*, *Pfu* or a blend of both (Marathon, Clontech) DNA polymerases were used at 40 U/ml under otherwise standard conditions. A further upstream sequence was cloned by 5′-RACE (Marathon, Clontech) on placenta cDNA, using the antisense gene-specific primer h2asR. Placenta Pol μ cDNA was completed at the 3′ end by specific PCR between primers h2sR and h2asZ. The placenta cDNA thus obtained contained an open reading frame (ORF) highly homologous to the TdT sequence but interrupted by a frameshift at position 687. The Pol μ sequence was confirmed on a Ramos cell line in which cDNA clones without a frameshift were found. Pol μ cDNA has a length of 2589 bp, with 45 bp of 5′-untranslated region, 1482 bp of coding sequence (494 amino acids) and 1062 bp of 3′-untranslated region. This sequence was submitted to the DDBJ/EMBL/GenBank databases under the accession No. AJ131891.

Chromosomal mapping

A preliminary mapping of human Pol μ was carried out by PCR screening of a panel of human–rodent somatic cell hybrids (BIOS Somatic Cell Hybrid PCRable DNAs, BIOS Laboratories, New Haven, CT). Following a 3 min denaturation step at 94°C, 40 cycles of amplification were performed: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCRs were performed in a total volume of 10 μl, using 25 ng of template, 5 μM primers h2MAPs and h2MAPas, 1.25 mM $MgCl_2$, 0.2 mM dNTPs and 0.025 U of *Taq* DNA polymerase (Gibco-BRL) in the buffer supplied by the manufacturer. No signal was detected from mouse and hamster genomic DNA using these amplification conditions. Using the same specific primers, h2MAPs and h2MAPas, a more precise chromosomal mapping of the *POLM* gene, coding for Pol μ, was carried out by PCR screening of the High Resolution Stanford TNG3 Radiation Hybrid Panel RH03.02 (Research Genetics, Huntsville, AL). The PCRs were performed as described above. Data were submitted to the Stanford Radiation Hybrid Server (rhserver@shgc.stanford.edu), which returned the linked data reported in Results.

Amino acid sequence comparisons

The initial alignment of Pol μ with TdT was done using the program MULTALIN (http://www.toulouse.inra.fr/multalin.html). As a second step, the alignment obtained was adjusted manually, and refined on the basis of the secondary structure elements of rat Pol β, as deduced from its crystal structure (Pelletier *et al*., 1994; Sawaya *et al*., 1994). A BRCT domain (Bork *et al*., 1997; Callebaut and Mornon, 1997), located between Pol μ residues 22 and 118, was predicted by searching the first 140 amino acids of Pol μ against the PROSITE library, using the program PROFILESCAN (http://www.isrec.isb-sib.ch/index.html).

Overproduction of human Pol μ in E.coli cells

The complete coding sequence corresponding to Pol μ was cloned into the pRSET-A bacterial expression vector (Invitrogen). The Pol μ complete ORF was amplified by RT–PCR from Ramos cells initially in two overlapping fragments (h2NdeATGs–h2asQ3 and h2sQ2–h2ER1TGAas) that were subsequently merged into a single full-length cDNA by PCR with the outer primers (h2NdeATGs–h2ER1TGAas). The amplified 1485 bp product, from initiation to stop codon, was cloned in pZERO (Invitrogen), verified by sequencing and subcloned in the *Nde*I–*Eco*RI sites of the expression vector pRSET (Invitrogen), resulting in the construct named pRSET-hPolμ. Expression of Pol μ was carried out in the *E.coli* strain BL21(DE3) pLysS under conditions to be described elsewhere.

Purification of human Pol μ

The *E.coli* cells expressing human Pol μ were ground with alumina and the resulting lysate was centrifuged for 15 min at 15 000 *g* to separate insoluble proteins (debris) from the soluble extract. The DNA present in the soluble extract was removed by polyethyleneimine (PEI) precipitation and the supernatant was precipitated with ammonium sulfate to 50% saturation to obtain a PEI-free protein pellet. The precipitate was subjected to phosphocellulose chromatography followed by HiTrap Heparin (Pharmacia Biotech) chromatography. The final fraction contained highly purified Pol μ (>95%) in soluble form. A more detailed

description of the purification procedure will be published elsewhere. When indicated, the final fraction of the heparin–Sepharose column was loaded onto a 5 ml glycerol gradient (15–30%) containing 20 mM Tris–HCl pH 8, 200 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT), and centrifuged at 62 000 r.p.m. (Beckman SW.50 rotor) for 24 h at 4°C. After centrifugation, 20 fractions were collected from the bottom of the tube, examined in Coomassie Blue-stained gels and tested for DNA polymerase activity on activated DNA.

DNA polymerization on activated DNA

The incubation mixture contained, in 25 μl, 50 mM Tris–HCl pH 7.5, either 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml bovine serum albumin (BSA), 13 nM [α-^{32}P]dATP and 250 ng of activated calf thymus DNA (Pharmacia) as a substrate. This assay was used to monitor Pol μ throughout the purification procedure. Alternatively, TdT (5 U) or Klenow (1 U) were used as a control of DNA polymerization. When indicated, different amounts of additional deoxynucleotides were also added. After incubation for the indicated times at 37°C, the reaction was stopped by adding 10 mM EDTA and the samples were filtered through Sephadex G-50 spin columns. The excluded volume, corresponding to the labelled DNA, was counted (Cerenkov radiation). Polymerization activity was calculated as the amount of incorporated dAMP.

DNA polymerization assays on defined DNA molecules

Terminal transferase activity was evaluated by using 5′-labelled oligonucleotides [P15, P19, oligo(dA)$_{15}$ or oligo(dT)$_{10}$] as substrates. The incubation mixture contained, in 25 μl, 50 mM Tris–HCl pH 7.5, 1 mM $MnCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, different concentrations of the dNTPs indicated, 3.2 nM 5′-labelled oligonucleotide and 20 ng of purified Pol μ (glycerol gradient fraction) or 2.5 U of TdT (41 ng). After incubation for the indicated times at either 30 or 37°C, the reactions were stopped by adding EDTA up to 10 mM. Samples were analysed by 8 M urea–20% PAGE and autoradiography. When indicated, terminal transferase activity on a blunt-ended primer terminus, obtained by hybridization of oligonucleotides P19 and P19c, was assayed under identical conditions. DNA-dependent polymerization was assayed on defined primer–template structures, obtained by hybridization of either oligo(dA)$_{15}$ to poly(dT), oligo(dT)$_{10}$ to poly(dA), or 5′-labelled P15 to the template oligonucleotide T15c+6. The incubation mixture contained, in 25 μl, 50 mM Tris–HCl pH 7.5, either 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 3.2 nM hybrid indicated in each case and the amount and concentration indicated of either purified Pol μ or the DNA polymerase indicated. To analyse the base specificity (nucleotide insertion fidelity) of Pol μ, oligonucleotide P15 was hybridized to four variants of the T15c+6 template oligonucleotide: T15c+6 (T), T15c+6 (G), T15c+6 (C) or T15c+6 (A), differing in the first template base. Nucleotide insertion into each hybrid structure (3.2 nM) was studied comparatively in the presence of either $MgCl_2$ (10 mM) or $MnCl_2$ (1 mM) as metal activator, by providing 20 ng of Pol μ and various concentrations of either the correct dNTP (up to 10 μM) or each of the three incorrect dNTPs (up to 100 μM). After incubation at 30°C in the presence of the indicated dNTPs, the reactions were stopped by adding EDTA, and the samples were either filtered through Sephadex G-50 spin columns and quantitated from the Cerenkov radiation, or analysed by 8 M urea–20% PAGE and autoradiography. Quantitation of autoradiographs was done by densitometric analysis of the band(s) corresponding to primer extension products.

Northern blotting

RNA blots containing 2 μg of poly(A)$^+$ RNA per lane of different human tissues (MTN and MTN-II blots, Clontech) were hybridized with a probe (derived from EST AA298793) containing 1141 nucleotides corresponding to the 3′ end of Pol μ cDNA. The probe was labelled by random priming (Rediprime II, Amersham) with [α-^{32}P]dATP (Amersham). Blots were pre-hybridized for 4 h and then hybridized overnight in Rapid-hyb buffer (Amersham) at 65°C. Blots were washed twice with 2× SSC–0.1% SDS at room temperature, twice with 1× SSC–0.1% SDS and twice with 0.1× SSC–0.1% SDS at 65°C, prior to autoradiography.

In situ hybridization

Digoxigenin (DIG)-labelled riboprobes were prepared using the DIG RNA (SP6/T7) labelling kit (Boehringer Mannheim). Antisense and sense probes were generated from a linearized pGEM-T Easy plasmid (Promega) containing 1200 nucleotides corresponding to the 5′ end of Pol μ cDNA, using SP6 or T7 RNA polymerases, respectively. When indicated, antisense and sense probes corresponding to the A-myb transcription factor were prepared and used as a positive control for centroblast populations. Human tissue slides were purchased from Novagen (Human Tissue Set I and Human Hematal and Immune Tissue Set). Hybridization and immunological detection of either sense or antisense RNA probes were carried out essentially as described (Braissant and Wahli, 1998). Slides were mounted using Aquatex (Merck) and visualized under a microscope.

Acknowledgements

We would like to thank to M.A.Rodríguez-Marcos and M.L.Gaspar for many helpful discussions and critical reading of the manuscript. This work was supported by grants from DGES (PB97-1192) and CAM (08.1/0044/98) to L.B.; CAM (08.1/0044.2/98) to A.B.; and by an institutional grant from Fundación Ramón Areces to Centro de Biología Molecular 'Severo Ochoa'. The Department of Immunology and Oncology (CNB) was founded and supported by Pharmacia and Upjohn, and Consejo Superior de Investigaciones Científicas. J.F.R. was a predoctoral fellow from the Ministerio de Educación y Ciencia. T.K., T.L.d.L. and M.G.-D. were postdoctoral fellows from the Comunidad de Madrid.

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Received January 5, 2000; revised and accepted February 3, 2000