DNA polymerase mu, a candidate hypermutase?

José F. Ruiz¹, Orlando Domínguez¹, Teresa Laín de Lera², Miguel García-Díaz¹,
Antonio Bernad² and Luis Blanco^{1*}

¹*Centro de Biología Molecular Severo Ochoa (CSIC-UAM), and* ²*Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología (CSIC), Universidad Autónoma, 28049 Madrid, Spain*

A novel DNA polymerase (Pol μ) has been recently identified in human cells. The amino-acid sequence of Pol μ is 42% identical to that of terminal deoxynucleotidyl transferase (TdT), a DNA-independent DNA polymerase that contributes to antigen-receptor diversity. In this paper we review the evidence supporting the role of Pol μ in somatic hypermutation of immunoglobulin genes, a T-dependent process that selectively occurs at germinal centres: (i) preferential expression in secondary lymphoid organs; (ii) expression associated to developing germinal centres; and (iii) very low base discrimination during DNA-dependent DNA polymerization by Pol μ , a mutator phenotype enormously accentuated by the presence of activating Mn^{2+} ions. Moreover, its similarity to TdT, together with extrapolation to the crystal structure of DNA polymerase beta complexed (Pol β) with DNA, allows us to discuss the structural basis for the unprecedented error proneness of Pol μ , and to predict that Pol μ is structurally well suited to participate also in DNA end-filling steps occurring both during V(D)J recombination and repair of DNA double-strand breaks that are processed by non-homologous end-joining.

Keywords: DNA polymerase mu; error-prone DNA polymerase; terminal deoxynucleotidyl transferase homologue; somatic hypermutation

1. INTRODUCTION

The stability of genetic information in DNA organisms is not only based on a faithful replication but also on the existence of multiple DNA repair pathways that efficiently correct most kind of DNA damage. On the contrary, it is becoming evident that there is an alternative enzymology that counteracts DNA repair, generating variability or bypassing damaged DNA templates (Friedberg & Gerlach 1999; Friedberg *et al.* 2000; Hübscher *et al.* 2000). One of the revealing examples on the existence of a specific enzyme involved in the generation of diversity is terminal deoxynucleotidyl transferase (TdT), although it selectively targets particular genes as those coding for antigen receptors (Komori *et al.* 1996).

We have recently identified and partially characterized a novel eukaryotic DNA polymerase in human cells named DNA polymerase mu (Pol μ) (Domínguez *et al.* 2000) of which beta amino-acid sequence is 42% identical to that of TdT. TdT is a peculiar member of the DNA polymerase X family (Ito & Braithwaite 1991), the structural paradigm of which is DNA polymerase beta (Pol β), specifically involved in DNA repair. Since that similarity value was significantly higher than that relating Pol μ and Pol β (23%) or that relating Pol β and TdT (22%), it was likely that Pol μ and TdT could share some functional analogy (described in §3). More recently, we have cloned the complete cDNA sequence corresponding to mouse Pol μ and have obtained evidence on the existence of bovine and swine Pol μ orthologues (J. F. Ruiz, O. Domínguez,

T. Laín de Lera, M. García-Díaz, A. Bernad and L. Blanco, unpublished data). The genomic locus corresponding to mouse Pol μ spans about 12 kb and, like that corresponding to human TdT, it contains 11 exons, in agreement with the evolutionarily relatedness of these two enzymes (J. F. Ruiz, O. Domínguez, T. Laín de Lera, M. García-Díaz, A. Bernad and L. Blanco, unpublished data).

The human gene (*POLM*) coding for Pol μ was mapped within chromosomal band 7p13, one of the four known fragile sites in lymphocytes with a high incidence of molecular alterations such as deletions, inversions and translocations (Domínguez *et al.* 2000).

2. DNA POLYMERASE MU IS PREFERENTIALLY EXPRESSED AT SECONDARY LYMPHOID ORGANS AND ASSOCIATED WITH GERMINAL CENTRES

TdT is a DNA-independent DNA polymerase that contributes to antigen-receptor diversity and whose expression is regulated both during development (absent in foetal and neonatal lymphocytes) and during B-cell differentiation (only present in proB cells; Gilfillan *et al.* 1995). Thus, at the tissue level, TdT expression is restricted to primary lymphoid tissues (thymus and bone marrow).

Unlike TdT, expression analysis of Pol μ mRNA (carried out by Northern blotting) indicated a predominant presence in secondary lymphoid tissues (lymph nodes and spleen), although basal levels were detected in most other tissues analysed, including thymus and bone marrow (Domínguez *et al.* 2000). A more detailed *in situ* analysis of Pol μ mRNA in human tissue sections showed

*Author for correspondence (l blanco@cbm.uam.es).

Figure 1. Pol μ mRNA expression in splenic keyhole-limpet haemocyanin (KLH)-induced GCs. Male FVB/N mice (6–12 weeks old) were immunized by injection of alumina-precipitated KLH (50 mg) together with complete Freund adjuvant. Mice were sacrificed 0–12 days following immunization; their spleens were removed, embedded in Tissue-Tek OCT (Baxter Scientific, Boston, MA, USA), frozen on dry ice and stored at -80°C . Digoxigenin (DIG)-labelled sense and antisense riboprobes, corresponding to the first 1200 nucleotides of murine Pol μ cDNA, were prepared using the DIG RNA (SP6/T7) labelling kit (Boehringer–Mannheim, Philadelphia, PA, USA). Cryostat sections ($8\mu\text{m}$) were mounted onto Fisherbrand Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), fixed in 4% paraformaldehyde–PBS for 20 min, and processed for *in situ* hybridization, essentially as described (Braissant & Wahli 1998). The specific signal was revealed using an alkaline phosphatase conjugated sheep anti-DIG antibody and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) (Boehringer–Mannheim). After extensive washing with PBS, the slides were further incubated with biotinylated peanut agglutinin (PNA) (Sigma Chemical, St Louis, MO, USA), diluted (1 mg ml^{-1}) in blocking solution (PBS–0.1%BSA), followed by incubation with streptavidine-alkaline phosphatase conjugate (Boehringer–Mannheim diluted (1/300) in blocking solution and visualized using 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride/BCIP substrate (Boehringer–Mannheim). Slides were air-dried and mounted using Aquatex (Merck, Darmstadt, Germany) and analysed under a light microscope. GCs, specifically stained with PNA, are identified as circular regions with an orange staining. The blue signal, observed with the antisense riboprobe, outlines regions largely expressing Pol μ mRNA that are clearly associated to GCs. No comparable signal was obtained by using the sense riboprobe under the same experimental conditions and in consecutive tissue sections (not shown).

an abundant signal associated with the follicular areas of secondary lymphoid tissues. Interestingly, and compatible with our expression data, a computer analysis of the expressed sequence tag (EST) database indicated that human Pol μ was significantly expressed in germinal centre (GC)-B cells, suggesting its participation in specific processes associated with these cells (Domínguez *et al.* 2000).

In agreement with all these expression data, we have recently demonstrated a direct correlation between Pol μ mRNA expression and the development of GCs in spleens from immunized mice. As shown in figure 1, the hybridization signal corresponding to an antisense cDNA probe specific for murine Pol μ (blue staining) was clearly detected in association with developing GCs appearing in the mouse spleen seven to ten days after keyhole-limpet

haemocyanin/aluminina immunization, co-localizing with the specific and characteristic (orange staining) peanut agglutinin (PNA)-staining of splenic GCs (Rose *et al.* 1980; Han *et al.* 1996). These results are also supported by the positive Pol μ immunostaining of GCs formed in human tonsils, and by reverse transcriptase (RT)-PCR detection of Pol μ at specific cell types (B220⁺/PNA^{high} centroblasts) purified by flow-sorting from immunized mouse spleens (J. F. Ruiz, O. Domínguez, T. Laín de Lera, M. García-Díaz, A. Bernad and L. Blanco, unpublished data).

The specific pattern of expression of Pol μ , and its association with GC-B cells, is perhaps the main argument supporting our hypothesis that Pol μ could be involved in the somatic hypermutation of immunoglobulin (Ig) genes (Domínguez *et al.* 2000), a T-dependent process that

Figure 2. Multiple alignment of human Pol μ , human TdT and human Pol β . Numbers between slashes indicate the amino-acid position relative to the N-terminus of each DNA polymerase. The first 140 amino acids of Pol μ and the first 152 amino acids of TdT, that have no counterpart in Pol β , contain a BRCT domain (blue area), frequently found in cell cycle checkpoint and DNA repair proteins (Bork *et al.* 1997). According to Pol β structural data (Pelletier *et al.* 1994; Sawaya *et al.* 1994), the rest of the alignment can be divided in four subdomains: '8 kDa' (Pol β residues 1–91; green area), 'fingers' (Pol β residues 92–148; yellow area), 'palm' (Pol β residues 149–260; red area), and 'thumb' (Pol β residues 261–335; magenta area). α -helices (lettered) and β -strands (numbered) are indicated at the top of the alignment. Two helix-hairpin-helix motifs (HhH) are between α -helices C and D ('8 kDa' subdomain), and between α -helices F and G ('fingers' subdomain). Invariant residues among Pol μ and TdT and/or Pol β are indicated with white letters (over a black background). Conservative substitutions (bold) were considered as follows: K, H and R; D, E, Q and N; W, F, Y, I, L, V, M, A; and G, S, T, C and P. The 27 Pol β residues that are invariant among DNA polymerase X members (Oliveros *et al.* 1997) are indicated with open circles above the sequence of Pol μ . Coloured dots at the bottom of the alignment indicate Pol β residues (Pelletier *et al.* 1994) shown to act as DNA ligands (Gly⁶⁴, Gly⁶⁶, Gly¹⁰⁵, Gly¹⁰⁹, Lys²³⁴, Arg²⁵⁴, Arg²⁸³, Tyr²⁹⁶; blue), dNTP and metal ligands (Phe²⁷², Gly²⁷⁴, Arg¹⁸³, Asp¹⁹⁰, Asp¹⁹² and Asp²⁵⁶; green). Pol β residues involved in interactions between the 'palm' and 'thumb' subdomains (Gly¹⁷⁹/Phe²⁷²; Arg¹⁸²/Glu³¹⁶) are indicated with squares. Yellow dots indicate Pol β residues (Lys³⁵, Tyr³⁹, Lys⁴¹, Leu⁶², Val⁶⁵, Lys⁶⁸) forming the DNA interaction surface present at the 8 kDa domain of Pol β (Matsumoto *et al.* 1998; Prasad *et al.* 1998a,b). The catalytic residue of Pol β (Lys⁷²) responsible for the dRPase activity (Matsumoto *et al.* 1998) is indicated with a black dot.

selectively occurs in GCs (Berek *et al.* 1991; Jacob *et al.* 1991). Importantly, although the molecular basis for the selective targeting of the hypermutation process in the V regions of the gene segments coding for Igs is still a matter of speculation, a candidate hypermutase must be endowed with a very low base discrimination in order to produce the minimal frequency of 10^{-3} to 10^{-4} point mutations per base pair per generation that is characteristic of the hypermutation process (reviewed by Neuberger & Milstein 1995). In § 3, we will summarize a preliminary analysis of the enzymatic properties of Pol μ that enable this enzyme to act as a DNA mutator.

3. ENZYMATIC ACTIVITY OF DNA POLYMERASE MU AND TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE: A COMPARISON

TdT is a DNA-independent DNA polymerase, i.e. has the ability to add nucleotides to DNA without any template information. This unusual capacity is exploited to generate diversity at the broken ends of the V(D)J recombination intermediates of rearranging antigen-receptor genes. Pol μ and TdT are close homologues and, accordingly, both are endowed with a DNA-independent TdT activity (Domínguez *et al.* 2000). Under similar assay conditions in which TdT preferentially inserts dGTP and dCTP, Pol μ displayed a much lower catalytic efficiency and a different nucleotide usage, preferentially inserting pyrimidine nucleotides (dT >> dC > dG > dA).

On the other hand, and unlike TdT, Pol μ is strongly stimulated (up to 370-fold) by the presence of a template strand. Therefore, and in spite of its intrinsic TdT activity, Pol μ may be defined as a DNA-dependent DNA polymerase since it requires a template primer for optimal activity. One of the first evidences of the error proneness of Pol μ was the fact that dAMP incorporation on Poly dT/oligo dA was strongly inhibited by relatively low concentrations of any of the other three (non-complementary) nucleotides (Domínguez *et al.* 2000). A preliminary analysis of misincorporation at different template bases allowed us to conclude that in the presence of Mg^{2+} ions, the insertion frequency of particular errors was only 10–50-fold lower than that of the correct nucleotide. Interestingly, the presence of activating Mn^{2+} ions accentuated this mutator phenotype, allowing Pol μ to catalyse random polymerization of nucleotides at different template bases (Domínguez *et al.* 2000).

All these results demonstrate that Pol μ is a DNA-dependent DNA polymerase with an unprecedented error proneness, which could be exploited for the generation of Ig diversity in GC-B cells. Further studies of the Pol μ hypermutator capacity will be required to establish the preferred and accessible DNA structures among DNA ends versus nicked, gapped, flapped or mismatched DNA, and to analyse its error specificity at those sequences known as hot spots or target sequences for somatic hypermutation (Levy *et al.* 1988; Betz *et al.* 1993, 1994; Smith *et al.* 1996; González-Fernández *et al.* 1994). It will be also interesting to test if the extent of the reaction and spectra of mutations will be influenced by the presence of DNA protein kinase, as has been reported to be the case for TdT nucleotidyl transferases (Mickelsen *et al.* 1999).

Potential structural–functional relationships orientated to determine the molecular basis of Pol μ mutagenicity, predicted by extrapolation to Pol β structure, are discussed in § 4.

4. FUNCTIONAL PREDICTIONS BASED ON EXTRAPOLATION FROM DNA POLYMERASE BETA STRUCTURE

Although structurally related, Pol μ , TdT and Pol β must have unique features that explain their different enzymatic activities. We have carried out the alignment of their amino-acid sequences and the extrapolation of both Pol μ and TdT sequences to the crystal structure of Pol β complexed with different DNA substrates. These studies are valuable to identify both generally conserved and specific residues potentially involved in either common or specific features of these related enzymes. Future mutational analysis of candidate residues is expected to provide a structural basis for the unprecedented error proneness of Pol μ .

The multiple alignment shown in figure 2 strongly indicates a close evolutionary relationship between Pol μ and TdT. Based on quantitation in the portion aligned, *ca.* 42% and 21% of Pol μ amino-acid residues align with identical residues in human TdT and human Pol β , respectively. Moreover, Pol μ conserves 146 out of the 216 (67%) amino-acid residues that are invariant among TdTs from different origins (red dots in figure 2). Interestingly, Pol μ contains 25 out of the 27 amino-acid residues (open circles in figure 2) that are invariantly present in all members (including also TdT) of the Pol *X* family (Oliveros *et al.* 1997). The functional importance of some of these residues are described in detail in the following sections.

(a) Modular organization

The resolution of the three-dimensional (3D) structure of Pol β demonstrated that its 335 amino acids are folded in two distinct domains, separated by a protease-sensitive region: a specific N-terminal '8 kDa' domain, implicated in DNA binding and deoxyribose phosphate excision, and a C-terminal 31 kDa domain, containing the polymerase catalytic site. The catalytic domain showed also the common fold of three distinct subdomains: 'fingers', 'palm' and 'thumb', characteristic of different polymerase super-families, previously shown in the Klenow fragment (KF) of *Escherichia coli* DNA polymerase I (Ollis *et al.* 1985), HIV-1 RT (Kohlstaedt *et al.* 1992; Jacobo-Molina *et al.* 1993) and T7 RNA polymerase (Sousa *et al.* 1993). The amino-acid sequence alignment shown in figure 2, adjusted on the basis of the Pol β 3D structure (see figure 4*b,c*), allows the prediction that Pol μ and TdT have a similar 3D organization constituted by the four subdomains: '8 kDa', 'fingers', 'palm' and 'thumb', that constitute an evolutionarily conserved Pol β core. Exceptionally, Pol μ and TdT contain a breast cancer 1 (BRCA 1) protein-C-terminal domain (BRCT) domain at its N-terminal domain (Domínguez *et al.* 2000) that is not present in Pol β .

(b) DNA binding and processivity

Four DNA ligands have been identified in Pol β (Pelletier *et al.* 1994) that map to the 'palm' and 'thumb' subdomains.

- (i) Lys²³⁴, located in the β -turn between strands three and four of the 'palm' subdomain and interacting through the phosphates and base of the template strand, is invariant in TdTs from different species and in most members of the Pol X family; exceptionally, this residue is substituted by an arginine residue in human Pol μ (Arg³⁸⁷), although it could play an analogous function. Both Pol μ and TdT have an insertion of about 14 amino-acid residues with respect to Pol β in the β -turn preceding this residue.
- (ii) Arg²⁵⁴, preceding β -strand five of the 'palm' subdomain and H-bonded to the phosphate of the nucleotide forming the primer terminus, is invariant in Pol μ and TdT.
- (iii) Arg²⁸³, located in α -helix N of the 'thumb' subdomain and H-bonded to the templating base, is invariant in Pol μ and TdT, and in all other members of the Pol X family.
- (iv) Tyr²⁹⁶, located on a loop between β -strands six and seven of the 'thumb' subdomain and interacting with the backbone phosphates of the template strand, is substituted for a histidine residue in Pol μ (His⁴⁵⁹) and TdT (His⁴⁷³).

Among these residues, Lys²³⁴ and Arg²⁸³ of Pol β were proposed to break up the water structure in the minor groove of the template primer upon complex formation, as the first step in the B-DNA to A-DNA transition at the Pol β active site (Pelletier *et al.* 1996).

In addition to these generally conserved DNA binding 'core' residues, resolution of the crystal structure of Pol β complexed with different DNA substrates allowed the identification of two structurally similar, but functionally distinct, DNA binding motifs named helix-hairpin-helix (HhH).

- (i) The HhH motif present in the '8 kDa' domain (formed by helices C and D, see figure 2) binds the 5'-phosphate end of a DNA gap (Prasad *et al.* 1994), allowing the removal of a 5'-deoxyribose phosphate (dRP) intermediate prior to the gap-filling step during the base excision repair (BER) reaction (Matsumoto & Kim 1995; Pelletier *et al.* 1996). Interestingly, both Pol μ and TdT conserve this HhH motif, together with most of the residues of the 8 kDa domain that form the enzyme-DNA interaction surface (yellow dots in figure 2). On the other hand, neither Pol μ nor TdT conserve the catalytic lysine residue (Lys72 in Pol β , black dot in figure 2) required to form the imine enzyme-DNA intermediate responsible for dRP release (Matsumoto *et al.* 1998). Pol β , a distributive enzyme on primer-template molecules, has been shown to be processive when filling short DNA gaps. Such a processive gap-filling by Pol β was structurally and functionally related to the presence of the '8 kDa domain' and strongly favoured by the presence of a 5'-PO₄ group at the end of the gap (Prasad *et al.* 1994). The possible implications of the 8 kDa domain for Pol μ function will be discussed in § 6.
- (ii) The HhH motif in the 'fingers' subdomain (formed by helices F and G, see figure 1) helps to position the primer strand of the DNA substrate in the

polymerase active site and its coordinated metal ion was proposed to act as a 'processivity cofactor' (Pelletier *et al.* 1996). On the basis of the amino-acid sequence alignment shown in figure 2, both Pol μ and TdT conserves this HhH motif, which is embedded in one of the most conserved segments among TdTs.

A comparative analysis of the structurally solved HhH motifs supports their role in non-sequence specific recognition of DNA, a feature that appears to be common in different proteins involved in DNA repair processes (Doherty *et al.* 1996) and that is also preserved in the structure of Pol μ .

(c) Nucleotide binding and catalysis

All classes of nucleic-acid synthesizing enzymes appear to have a similar 3D arrangement of three acidic residues contained in the generally conserved motifs A and C (Delarue *et al.* 1990). These residues, always located in the 'palm' subdomain, form a carboxylate triad implicated in the arrangement of a pair of divalent metal ions in a precise geometry to catalyse the nucleotidyl transfer reaction. In Pol β , these residues are Asp¹⁹⁰, Asp¹⁹² and Asp²⁵⁶. As shown in figure 1, these three aspartates correspond to residues Asp³³⁰, Asp³³² and Asp⁴¹⁸ of human Pol μ . As expected, these residues are located in two segments with the highest similarity to TdT.

Much of the nucleotide binding site observed in the ternary complex of Pol β is made up of the 3'-terminus of the primer strand and the template overhang. Additionally, the two metal ions in the Pol β site bind to all three phosphates (α , β , γ) of the incoming nucleotide. However, two Pol β residues interact by van der Waals contacts with the sugar moiety (C2' and C3' carbons) of the nucleotide: Phe²⁷² and Gly²⁷⁴, located at the end of α -helix M and predicted to participate in nucleotide selectivity of DNA over RNA. This glycine residue is invariant in both Pol μ (Gly⁴³⁶) and TdT (Gly⁴⁵¹), as in all other members of the Pol X family. However, the phenylalanine residue is substituted by a tryptophan in Pol μ (Trp⁴³⁴) and TdT (Trp⁴⁴⁹). Moreover, Pol β residue Arg¹⁸³, located in α -helix K of the 'palm' subdomain and H-bonded to the negatively charged phosphate moiety of the nucleotide, is invariant in all Pol X members, including TdT (Arg³³⁶) and Pol μ (Arg³²³). It is conceivable that additional stabilizing contacts with the nucleotide could be provided by specific residues in Pol μ and TdT, making nucleotide insertion not strictly template dependent.

(d) Conformational changes

It has been shown that the 'thumb' subdomain of Pol β undergoes a conformational change triggered upon nucleotide binding (Pelletier *et al.* 1996). The *cis*-peptide bond found between Gly²⁷⁴ and Ser²⁷⁵ (between α -helices M and N of the 'thumb' subdomain) is critical for such a change from the more open conformation of the binary complex (enzyme-DNA) to the more closed conformation of the ternary complex (enzyme-DNA-dNTP). It has been also proposed that a change back to the open conformation after nucleotide addition could be critical to facilitate pyrophosphate release after catalysis (Pelletier *et al.* 1996). In addition to numerous hydrophobic

proposed functions for Pol μ
antigen receptor variability and double-strand-break (DSB) repair

Figure 3. Proposed functions for Pol μ . A specific role of Pol μ in somatic hypermutation is proposed on the basis of its preferential expression in GCs formed at peripheral lymphoid tissues, together with its unprecedented error proneness. The structural similarity with TdT, outlined by the presence of a BRCT domain at the N-terminus of both enzymes, the presence of an independent DNA binding domain ('8 kDa') and the basal expression of Pol μ in most tissues, including thymus and bone marrow, support the proposal of a role for Pol μ in both specific and general DNA end-joining reactions associated to V(D)J recombination and DNA repair via non-homologous end-joining (NHEJ). The scheme showing the common partners operating at these two processes has been adapted from a previous publication (Critchlow *et al.* 1997).

interactions lining the 'palm'–'thumb' interface, a salt bridge between Pol β residues Arg¹⁸² (located in α -helix K) and Glu³¹⁶ (located in α -helix O) stabilizes the 'open-thumb' position. On the contrary, the 'closed-thumb' position is stabilized by an hydrogen bond between Gly¹⁷⁹ and Phe²⁷². As shown in figure 2, three of these residues are invariant in both Pol μ (Arg³²²; Glu⁴⁷⁶; Gly³¹⁹) and TdT (Arg³³⁵; Glu⁴⁹⁰; Gly³³²), whereas the phenylalanine residue is conservatively changed to tryptophan in both Pol μ (Trp⁴³⁴) and TdT (Trp⁴⁴⁹). The conservation of the critical residues referred above suggests that a mobile 'thumb' subdomain analogous to that of Pol β is operating also in Pol μ and TdTs during each nucleotidyl transfer cycle.

The general conservation of the Pol β core among functionally different enzymes as Pol β , Pol μ and TdT suggests that more subtle differences at the active site, even restricted to single amino-acid residues, could be responsible for the intrinsic properties of these enzymes regarding nucleotide insertion fidelity and template dependence. Additionally, these catalytic properties could be further modulated by specific interactions with accessory proteins. More evident differences, as the presence of additional domains (BRCT), could affect the enzyme processivity on physiologically relevant substrates or serve to interact with accessory proteins in order to be recruited

at specific sites. Crystallographic studies oriented to determine the 3D structure of Pol μ , together with a detailed mutational analysis at individual residues, are being carrying out in collaboration with Dr W. Young (National Institutes of Health, Bethesda, MD, USA). Both approaches will be critical to understand the molecular basis of the mutator capacity of Pol μ .

5. ALTERNATIVE ROLES OF DNA POLYMERASE MU IN ERROR-PRONE DNA REPAIR AND RECOMBINATION

Human Pol μ mRNA is preferentially expressed in lymphoid tissues, but is also present ubiquitously in many others, raising the possibility that this mutator polymerase could be involved in general processes either contributing to somatic variability, or requiring an error-prone DNA polymerase. As shown in figure 2, in addition to containing a conserved Pol β core, Pol μ and TdT have an homologous BRCT domain at their N-terminal portion. This domain, whose name derives from its initial finding at the C-terminal domain of breast cancer 1 (BRCA1) protein, is present in several proteins involved in DNA repair and cell-cycle checkpoint control, as Rad 4 and RAD 9 proteins from yeast, p53 binding protein and

Figure 4. (a) Modular organization of DNA polymerases from family X. The amino-acid sequence of each DNA polymerase (proportionally scaled) is represented by a bar, with the N-terminus at the left. The light blue coloured segment at the N-terminus represents the BRCT domain (present only in yeast Pol IV, Pol λ, Pol μ and TdT), that is proposed to be involved in protein–protein interactions. A conserved Pol β core is formed by the linear arrangement of four subdomains: 8 kDa (green); ‘fingers’ (F, yellow); ‘palm’ (P, red) and ‘thumb’ (T, magenta). In yeast Pol IV and Pol λ, the portion connecting the BRCT domain and the 8 kDa subdomain has an insertion of about 100 amino acids (coloured in grey) that is rich in serine and proline residues (García-Díaz *et al.* 2000). Interestingly, the most conserved structural elements that form the ‘palm’ subdomain (red boxes) are separated by intervening sequences (coloured in white) of variable length among the different members of family X. Considering this feature, it is very apparent that the closest homologue to Pol μ is TdT, whereas the closest homologue to the recently identified Pol λ (García-Díaz *et al.* 2000) is Pol β, although the former contains two additional domains at its N-terminus. The location of the three aspartates that form the catalytic triad is indicated with asterisks in the ‘palm’ subdomain. Due to its similar length in most DNA polymerases, the 8 kDa and ‘fingers’ subdomains were used as an alignment axis. African swine fever virus (ASFV) Pol X is the smallest member of this family that contains a minimal Pol β core constituted by only the ‘palm’ and ‘thumb’ subdomains (Oliveros *et al.* 1997). (b) 3D-structure of human Pol β complexed with nicked DNA. The four different subdomains that form a Pol β-like fold are coloured as in part (a) and also in figure 2: ‘8 kDa’ (green), endowed with a dRPase activity and providing additional DNA binding capacity, which stabilizes a ‘kinked’ conformation of the nicked DNA substrate (represented in white ‘balls and sticks’ model); ‘fingers’ (yellow), providing contacts to handle the DNA primer strand through its helix–hairpin–helix (HhH) motif; ‘palm’ (red), constituting an appropriate surface to accommodate the substrate molecules (nucleotides and DNA) and to present the pair of metal ions adequately for catalysis; ‘thumb’, providing template selectivity and reaction cycling via conformational changes. (c) Rotation (90° counter-clockwise) of the model shown in (b). (b,c) Generated using the SwissPDBviewer program, and the crystallographic data corresponding to PDB entry 1BPZ.

retinoblastoma (Bork *et al.* 1997). The BRCT domain, whose 3D-structure has been recently solved (Zhang *et al.* 1998; Thornton *et al.* 1999), has been shown to be involved in protein–protein interactions (Taylor *et al.* 1998; Yu *et al.* 1998; Chai *et al.* 1999; Soulier & Lowndes 1999). Recently, it has been demonstrated that the BRCT domain of TdT is required for a stable interaction with Ku70 subunit of

the DNA-dependent protein kinase (DNA-PK), a protein involved in both V(D)J recombination and non-homologous end-joining (NHEJ) of double strand break (DSB) (Mahajan *et al.* 1999). Taking into account the high similarity of TdT and Pol μ also at their BRCT domains, it is likely that Pol μ is able to interact with the Ku70 subunit of the DNA-PK. Thus, as depicted in

figure 3, in addition to a more specific role in somatic hypermutation, Pol μ could play a more general role in processes mediated by the Ku70 subunit, as follows.

- (i) New episodes of V(D)J recombination, as those described to occur in germinal centre B cells (Han *et al.* 1997; Papavasiliou *et al.* 1997); it has been proposed that the ability to alter Ig expression by new episodes of V(D)J recombination in the selective environment of the germinal centre may be an additional mechanism for inactivation or diversification of immune responses (Papavasiliou *et al.* 1997); the potential role of Pol μ would be based on its intrinsic terminal transferase activity, and on the fact that TdT appears not to be expressed in germinal centre B cells. Moreover, and in agreement with its presence in thymus and bone marrow, Pol μ could be required during normal V(D)J recombination as a DNA-dependent polymerase involved in the generation of the palindromic (P) sequences at coding ends, or during the gap-filling reactions required to couple N addition by TdT with the generation of ligatable DNA ends.
- (ii) The error-prone repair of those DSBs that are processed via Ku protein, a major pathway of DSB repair in mammalian cells (reviewed by Kanaar *et al.* 1998; Critchlow & Jackson 1998; Karran 2000) that does not require extensive homology between the DNA ends to be joined, and thus is named NHEJ. Several proteins have been identified as components of NHEJ (DNA-PKcs; Ku70; Ku80; Xrcc4; DNA ligase IV), allowing the proposal of general models for NHEJ in which the need for a DNA polymerase activity is frequently not taken into account. However, the development of *in vitro* systems, together with sequence analysis of different junctions processed by NHEJ, clearly indicated the occurrence, in most cases, of non-templated insertions and deletions that cannot be merely explained by the only action of nucleases and ligases at the rejoining ends (Labhart 1999). Interestingly, NHEJ is not only used for the repair of sporadic DSBs but is also the mechanism that processes the programmed DSBs during V(D)J recombination. During the latter, TdT, a specific DNA synthesizing enzyme, participates in adding non-templated nucleotides at the ends of the recombining intermediates, thus modifying the DNA information. Recent results suggest that TdT is recruited to the DNA ends via a specific interaction with both Ku70 that requires the BRCT domain of TdT and Ku80 subunits (Mahajan *et al.* 1999). Moreover, it appears that this interaction could also serve to modulate the processivity and nucleotide insertion specificity of TdT (Mickelsen *et al.* 1999). It is likely that Pol μ (which is 42% identical to TdT, and possesses an homologous BRCT domain at its N-terminus) therefore could be recruited to the DNA ends during NHEJ, modifying the terminal DNA sequences to facilitate the ultimate DNA ligation step. Such a potential role of Pol μ in NHEJ would be compatible with its general expression in most tissues, and could even be facilitated by both

its residual terminal transferase activity and its intrinsic error proneness when inserting nucleotides in front of a template. It has been recently shown that both transfected TdT and Ku70 co-localize, forming nuclear foci in non-lymphoid cells (not expressing TdT) upon induction of DSBs (Mahajan *et al.* 1999). It will be interesting to see if Pol μ is the TdT-like enzyme involved in a physiological interaction with this subunit of the DNA-PK in non-lymphoid cells.

- (iii) Following the same argumentation, Pol μ could participate in class-switch recombination of rearranged IgG genes, a process that occurs exclusively in B lymphocytes located at the marginal zone of GCs, and involves the two Ku subunits of DNA-PK (Zelazowski *et al.* 1997; Manis *et al.* 1998; Casellas *et al.* 1998). Although the underlying mechanisms are poorly understood, it has been suggested that switch recombination is a specialized recombination system that occurs through a DNA end-joining mechanism (Kenter & Wuerffel 1999) and it could therefore require a specific DNA polymerase.
- (iv) Although translocations in leukaemic cells appear to involve classical NHEJ junctions (Gillert *et al.* 1999), it has been recently reported that the particular translocation t(14;18) associated with follicular lymphomas presents a new type of nucleotide insertion at the Bcl-2/IgH break-points, which suggests the presence of a short-patch DNA synthesis, templated and error prone, embedded in N-regions (Jäger *et al.* 2000). It is tempting to speculate that the dual DNA-dependent error-prone polymerase and terminal transferase activities of Pol μ could be responsible for the generation of these insertions (B. Nadel, personal communication).

In §6, and based on the extrapolation to the crystal structure of Pol β (the structural paradigm of the Pol X family) complexed with a gapped or nicked DNA molecule (Sawaya *et al.* 1997), we discuss why both Pol μ and TdT are well suited structurally for the alignment of two DNA ends, an essential requirement for both NHEJ and V(D)J recombination.

6. WHAT IS THE ROLE OF THE 8 kDa DOMAIN IN DNA POLYMERASE MU?

The DNA polymerase X family of DNA polymerases is a peculiar group that includes: vertebrate Pol β , involved in DNA repair; bacterial Pol β -like enzymes (Aravind & Koonin 1998); yeast polymerase IV (POL4), enzymatically similar to Pol β (Prasad *et al.* 1993); mitochondrial Pol β from protozoans (Torri & Englund 1995); fission yeast hypothetical protein SpAC2FT, a putative POL4 homologue; TdT; and African swine fever virus (ASFV) Pol X, a 20 kDa enzyme constituted by a minimal Pol β core (Oliveros *et al.* 1997). As shown in figure 4a, all known members of the Pol X family, with the exception of the mini-polymerase β coded by ASFV, contain the four subdomains that form Pol β : 8 kDa (green), 'fingers' (yellow), 'palm' (red) and 'thumb' (magenta). Interestingly, whereas the main differences in size correspond to the 'palm' subdomain (containing the polymerase active

site residues), the length of the 8 kDa and 'fingers' sub-domains are almost identical in all cases.

According to both biochemical and structural studies of Pol β co-crystallized with gapped and nicked DNA (Sawaya *et al.* 1997), it has been shown that the 8 kDa domain has intrinsic DNA binding capacity, changing conformation upon DNA binding and making contact with the downstream (relative to the direction of polymerization) portion of the gapped or nicked strand. Such a contact with the 8 kDa domain appears to be strong enough to maintain not only a small-gapped DNA but even a nicked DNA, in a 'kinked' conformation (see figure 4*b,c*). This distorted structure could be catalytically competent only if it allows nucleotide binding at the active site upon translocation of both primer and template strands. The structural constraints for this active positioning, compatible with a one-nucleotide gap, but not with a nicked DNA (figure 4*b,c*) are in agreement with the very low capacity of Pol β to carry out strand displacement during gap-filling synthesis.

NHEJ involves a synapsis step in which the two ends are maintained in proximity, processing steps in which nucleases and polymerases act on the ends, and a ligation step. Due to the wide array of alternative substrates and products, it is conceivable that this process involves a DNA polymerase able to use microhomologies at the joining ends, as it has been recently proposed to occur in yeast (Wilson & Lieber 1999), and also endowed with the capacity of aligning the two DNA ends (King *et al.* 1994; Islas *et al.* 1998). The presence of an 8 kDa domain in Pol μ could provide this enzyme with a DNA binding domain different and separated from that forming the DNA primer cleft, which in addition to increasing stabilizing contacts during gap-filling synthesis could also be exploited to bridge the 'gap' during non homologous end-joining of two DNA ends (King *et al.* 1994). Similarly, the presence of this domain in TdT could be used to co-ordinate terminal addition of N nucleotides with the joining (bridging) of the two DNA ends generated during V(D)J recombination.

7. MODELS FOR SOMATIC HYPERMUTATION: WHAT IS THE SUBSTRATE FOR THE HYPERMUTASE?

The original model proposed that somatic hypermutation is triggered by the generation of single- or double-strand breaks in the V region of Ig genes, followed by an error-prone repair mechanism (Brenner & Milstein 1966). More recently, it has been demonstrated that such DNA strand breaks are specifically scattered within the V mutation domain, and that they are accessible to TdT when this enzyme is transfected in Ramos cell lines (Sale & Neuberger 1998). In agreement with the underlying transcription dependence of somatic hypermutation (see a review by Storb *et al.* 1998) it has been recently proposed that these breaks could be generated by structure-specific nucleases that would cleave stable R loops generated by transcription (Tian & Alt 2000). It is tempting to speculate that Pol μ , a TdT-like enzyme which is expressed at GCs, is recruited at these specific breaks, perhaps via protein-protein interactions through its BRCT domain. Recent results indicate that Pol μ is able to catalyse nucleotide insertions at a nicked DNA, a non-instructional

substrate that is non-functional for most DNA polymerases (J. F. Ruiz, O. Domínguez, T. Laín de Lera, M. García-Díaz, A. Bernad and L. Blanco, unpublished data). A detailed *in vitro* analysis of the error specificity of Pol μ at these single-strand breaks will be required to be compared with the *in vivo* patterns of somatic hypermutation.

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